University of Alberta

Analysis of methanogenic microbial communities from oil sands processing tailings

by

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Abstract

Tailings management in the oil sands industry aims to increase the rate of densification of fine clay and silt particles, releasing pore water for reuse in bitumen extraction and producing mature fine tailings (MFT) for subsequent use in reclamation. Microbial methane production has accelerated densification of the MFT, and acetate amendment may enhance this process. To identify important microorganisms in methane production, 16S rRNA gene sequences were used. At 22°C, methanogenic cultures from acetate-amended or unamended tailings comprised hydrogenotrophic methanogens and clostridial homoacetogens, regardless of acetate amendment. These groups could be participating in syntrophic acetate oxidation. In MFT samples collected from various depths of two active tailings ponds at the Syncrude Canada Mildred Lake site most methanogens were related to acetoclastic *Methanosaeta* spp. Most Bacteria were related to the Proteobacteria, including sulfate-reducing bacteria and hydrocarbon degraders. Based on these results, a possible pathway for methane production from MFT is proposed.

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1 Introduction

1.1 Oil Sands Tailings

1.1.1 The Oil Sands Industry

Alberta contains the largest oil sands reserves in the world, covering nearly 78,000 km^2. These reserves contain an estimated 1.7 trillion barrels of bitumen, and about 300 billion barrels of bitumen are recoverable using current technology. Raw bitumen is a thick, black oil, and can be upgraded to produce a synthetic crude oil (Agecoutay, 2003). The oil sands deposits in Alberta provide 34% of the total production of crude oil in Canada. Syncrude Canada, Ltd. (hereafter referred to as Syncrude) is one company currently mining the oil sands in Northern Alberta. In 2002, Syncrude produced 230,000 barrels of synthetic crude oil/d, with total annual production of 83.8 million barrels (Chalaturnyk et al., 2002).

The oil sands consist of sand, clay, bitumen and water, and exist as a black, asphalt-like solid. Water makes up about 4% of the oil sands by weight, surrounding each sand particle (Figure 1-1). This layer of water makes it possible to separate the bitumen from the sand using hot water. The bitumen was formed from organic Cretaceous shales in the southern part of the Alberta Sedimentary basin (Agecoutay, 2003).

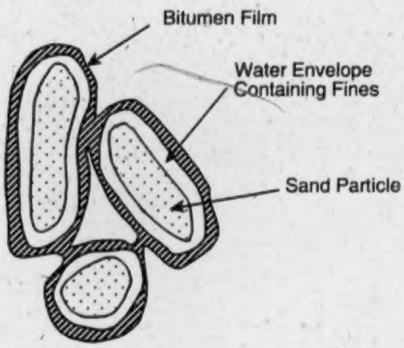

Figure 1-1: Schematic of the arrangement of oil sands particles. Adapted from Camp (1977).

Syncrude employs the truck and shovel method of mining the oil sands, as well as a process called hydrotransport, where the oil sands are mixed with water at the mine site to form a slurry, and pumped via pipeline to the extraction plant (Agecoutay, 2003). At the extraction plant, the oil sands are mixed with hot water, steam and caustic soda to reach a pH of 9 to 11 (Camp, 1977; FTFC, 1995; Schramm et al., 2000). Treatment with NaOH causes the asphaltic acids in the bitumen to act as surfactants and reduce the surface and interfacial tension in the oil sands slurry, which allows the bitumen to be separated from the sands (Chalaturnyk et al., 2002). Initial separation takes place in a primary separation vessel, where a bitumen "froth" forms at the surface, and the sand settles to the bottom. The middlings consist of suspended clay and silt fines, as well as some residual bitumen (Camp, 1977), and this fraction is transported to the tailings oil recovery process, which employs large cone-shaped vessels to separate out additional bitumen from the clay and silt fraction. The waste product of the extraction process is called tailings, and consists of the sand removed in the primary separation process, the silt and clay particles removed in the tailings oil recovery, as well as the water that is separated from the bitumen (Figure 1-2) (MacKinnon, 1989; Agecoutay, 2003).

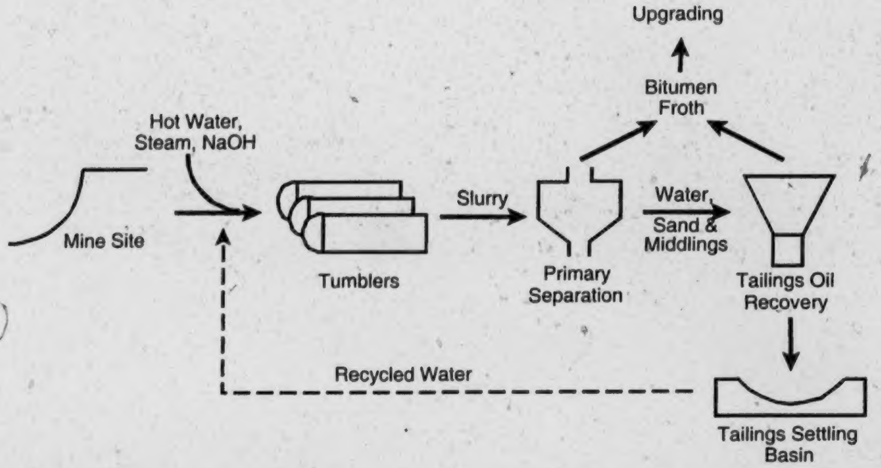
Upgrading
Bitumen Froth
Hot Water, Steam, NaOH
Mine Site
Tumblers
Slurry
Primary Separation
Water, Sand & Middlings
Tailings Oil Recovery
Recycled Water
Tailings Settling Basin

Figure 1-2: Illustration of the extraction process of bitumen from the oil sands. Adapted from Agecoutay (2003).

At Syncrude, the main storage site for fluid tailings is MLSB, which was established in 1978. The resulting pond covers an area of 10-13 km^2 (Fedorak et al., 2002). Tailings from the extraction process are pumped into MLSB at a rate of 347 000 t/d (List and Lord, 1997). When released from the plant, the tailings consist of approximately 45% water, 55% solids, and 0.5% residual bitumen. The solids fraction is 82% sand and 17% fines (< 44 μm diameter) (Chalaturnyk et al., 2002). The sand quickly settles out and forms a sloping underwater beach. About half of the fine tailings settle quickly or are trapped in the voids of the coarse tailings (Kasperski, 1992). The remaining fine tailings form a suspension consisting of about 7 to 10% solids. Consolidation, or densification, of the tailings occurs as water is slowly released from the particle suspension, which reaches 10 to 15% solids after several weeks and 20% solids after 3 to 9 months. After this, the consolidation occurs much more slowly, requiring 5 to 10 y to reach 25 to 30% solids (MacKinnon, 1989). When the tailings reach 30% solids, they are called mature fine tails (MFT) (Chalaturnyk et al., 2002) and consolidation of MFT beyond 30% was projected to require many decades (MacKinnon, 1989). The tailings are predicted to require 125 to 150 y to reach a density that is appropriate for use in land reclamation (Eckert et al., 1996).

The reason that the tailings are so stable is a side-effect of the NaOH used in the bitumen extraction. The reduction in interfacial tension of the tailings suspension allows the clay particles to remain suspended and to form a stable, gel-like structure (Kotlyar et al., 1992; Chalaturnyk et al., 2002). The slow densification results in a stratified pond, with the top 3 m consisting of relatively clear water that can be recycled back into the extraction process (Figure 1-3). The interface between the water and the tailings is very defined, with an increase in solids content from less than 0.1% to greater than 10% occurring over a depth difference of less than 0.3 m (MacKinnon, 1989). Beneath the clarified water is a zone of "immature" tailings which are in the process of consolidation. This zone can be up to 40 m thick in some areas (Lawrence et al., 1991). The solids layer increases from about 10% solids at the tailings:water interface to 50% solids at the greatest depths.

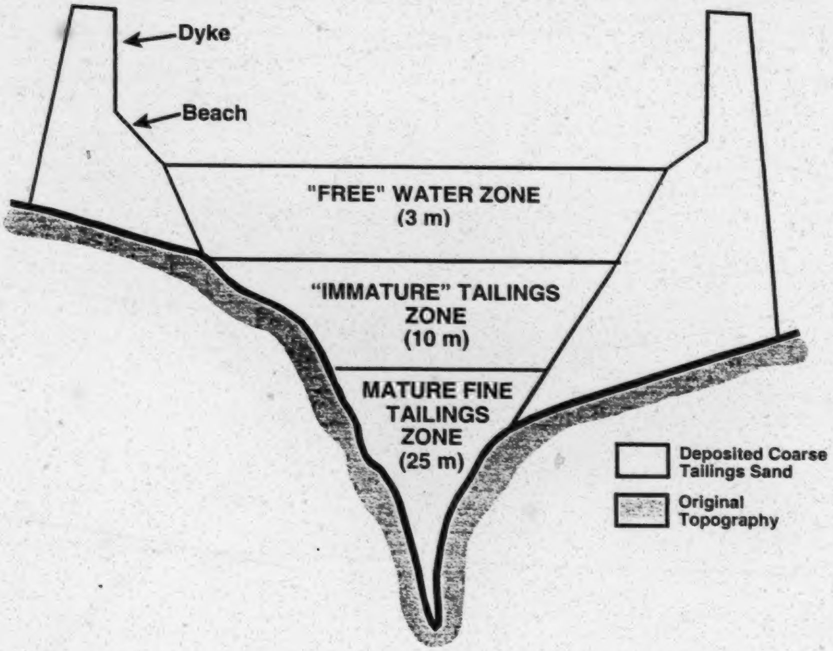
Dyke
Beach
"FREE" WATER ZONE
(3 m)
"IMMATURE" TAILINGS
ZONE
(10 m)
MATURE FINE
TAILINGS
ZONE
(25 m)
Deposited Coarse Tailings Sand
Original Topography

Figure 1-3: Cross section of MLSB. The vertical scale is greatly exaggerated. Adapted from MacKinnon (1989).

1.1.2 Densification Strategies

The settling basins on the Syncrude lease site currently contains more than 400 million m^3 of fine tailings (Fedorak et al., 2002). These tailings must be disposed of after Syncrude's lease expires and mining of the oil sands ceases (Kasperski, 1992). Because each cubic meter of oil sands produces about 4 m^3 of tailings material, simply replacing the tailings back into the mined-out pits is not possible without some type of treatment (Fedorak et al., 2003). The densification of the fluid fine tailings must occur before they can be used in land reclamation, because densification reduces the volume of the tailings that require storage, as well as increasing the strength of the tailings.

Two methods for accelerating the formation of tailings suitable for reclamation are based on engineered tailings options using divalent cations (Ca^{2+}) or organic flocculent aids (polyacrylamide). Other methods include raising or lowering the pH to a range that destabilizes the gel structure, bacterial treatment, freeze-thaw dewatering, and using high-intensity sound waves or electrophoretic fields to increase settling (Chalaturnyk et al., 2002). These methods vary in their economic feasibility and practicality for use on a large scale.

Treatment with Ca^{2+} to form consolidated tailings (CT) is currently being employed by Syncrude (MacKinnon et al., 2001). The gel structure of the tailings is destabilized by adding divalent calcium ions in the form of gypsum ($CaSO_4 \cdot 2H_2O$) (Sworska et al., 2000) or lime ($Ca(OH)_2$) (Chalaturnyk et al., 2002) to form CT. This suspension consolidates to 62% to 70% solids a few hours after the addition of the calcium, and the CT can eventually be used in land reclamation. It has been found that lime is a better flocculent than gypsum, but gypsum is less expensive, so gypsum is currently being used as the source of Ca^{2+} by Syncrude (Chalaturnyk et al., 2002). The released water is has elevated dissolved Ca^{2+}, which can interfere with the bitumen extraction process when the water is recycled back into the extraction plant, requiring more NaOH to be added to separate the bitumen (Chalaturnyk et al., 2002). This may eventually require treatment of the clarified water to remove the Ca^{2+} before water can be recycled back into the extraction process, or may make it necessary to take additional water from the Athabasca River for bitumen extraction.

Current research is investigating the use of a charged polymer such as polyacrylamide to aid in the densification process. Polyacrylamide is added to flocculate the clay particles. It is thought that the charged sidechains on the polyacrylamide polymer adsorb to the surface of many clay particles and pull them together, allowing the formation of large “flocs” which settle quickly (Sworska et al., 2000). This process creates a stable paste that, if it densifies sufficiently, may allow for faster reclamation (Kessick, 1981).

Recently, microbial methane production has been found to increase the densification of MFT, both in situ and in laboratory studies. This is discussed in more detail below (Section 1.2.2.1) and is the main focus of this thesis.

1.1.3 Reclamation Strategies

Two strategies are being considered for use of the tailings in land reclamation. The first is the "wet landscape approach," involving pumping the MFT to a mined-out pit that is eventually covered with water to create a lake or pond (Figure 1-4). A natural freshwater ecosystem will eventually be established in and around the pond (FTFC, 1995; List and Lord, 1997). Wet landscape reclamation demonstration ponds were created by Syncrude in 1989 and 1993. The water in the cap layer above the matured fines maintained low suspended solids with only minor alterations in inorganic and organic composition due to the presence of the MFT layer. A healthy ecosystem became established with diverse and abundant aquatic plant and animal communities growing in and around the ponds. The MFT zone in water capping scenarios is expected to continue to consolidate after transfer to a final secure reclamation site and may impact the quality of the water cap water cap but still allow development of viable and self-sustaining aquatic environments (List and Lord, 1997).

A second reclamation strategy referred to as the "dry landscape approach" involves the incorporation of soft fluid tailings into solid deposits that are eventually revegetated, and a natural ecosystem is allowed to become established (FTFC, 1995; List and Lord, 1997). The use of engineered tailings, such as CT in "dry landscape" reclamation is also being investigated (Fedorak et al., 2002).

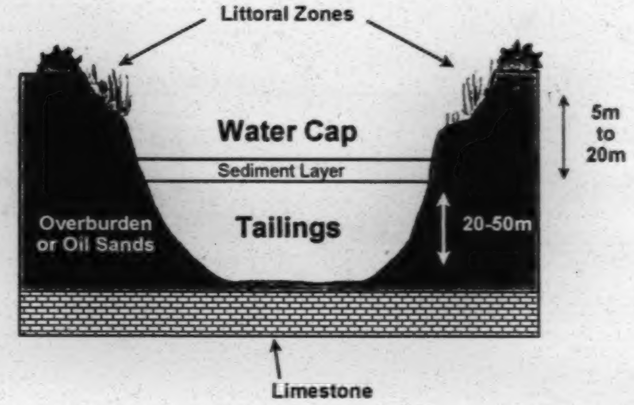

Figure 1-4: Schematic of a water-capped lake in the "wet landscape approach."

1.2 Anaerobic Microbial Activity in Aquatic Environments

1.2.1 Microorganisms in Anaerobic Environments

Anaerobic environments contain many different groups of microorganisms, which use different electron acceptors in place of oxygen. These can include nitrate-, iron-, and sulfate-reducers, which reduce NO_3^-, Fe^{3+} and SO_4^{2-} respectively. Also present in anaerobic environments are methanogens, which reduce CO_2 to methane. Respiration using oxygen as a terminal electron acceptor yields the highest amount of energy, because oxygen has the highest redox potential. Oxygen is not present in anaerobic environments, so the electron acceptors with the next highest redox potential are NO_3^-, Fe^{3+}, and SO_4^{2-}. The first two are not present in high concentrations in most anaerobic aquatic environments and therefore cannot support nitrate and iron reducers (Atlas and Bartha, 1998). When SO_4^{2-} is present, sulfate-reducing bacteria (SRB) have a competitive advantage over methanogens, because SRB can obtain more energy from sulfate reduction and compete more efficiently for electron donors such as hydrogen. However, as sulfate becomes depleted in aquatic anaerobic sediment with depth, SRB do not have sufficient energy sources and the methanogenic populations have an opportunity to become established (Raskin et al., 1996; Atlas and Bartha, 1998). Some SRB can also switch to fermentative metabolism when sulfate is depleted, and degrade larger organic molecules to provide substrates for methanogens. In some environments, the interactions between methanogens and SRB are not as straightforward. Methanogens can out-compete SRB in certain situations, for example, methanogens can become dominant at very high substrate concentrations in high-rate anaerobic reactors, even if sulfate is present (Isa et al., 1986). This is possibly because methanogens have a higher growth rate than do SRB when high acetate concentrations are present and prevent competition for this substrate (Yoda et al., 1987).

Organic matter is degraded in anaerobic environments by several different types of microorganisms (Figure 1-5). First, hydrolytic bacteria cleave polymers, such as starches, proteins, and nucleic acids, into their monomeric subunits, and subsequently ferment the monomers into a variety of smaller organic acids and alcohols, as well as hydrogen and carbon dioxide. Acetogenic bacteria can then convert these acids and alcohols into one- or two-carbon compounds, such as acetate, formate, and carbon dioxide, as well as releasing H_2. All of these small compounds are substrates for methanogens and homoacetogens. Homoacetogens are characterized by the ability to reduce CO_2 with H_2, allowing them to grow either autotrophically on CO_2 and H_2, or to ferment sugars releasing only acetate (Lay et al., 1998). The CO_2 normally reduced by glycolysis is subsequently reduced with H_2 to acetate, allowing homoacetogens to obtain more energy from the fermentation of sugars. Methanogens carry out the final steps in the degradation of organic matter, and release methane as a final product.

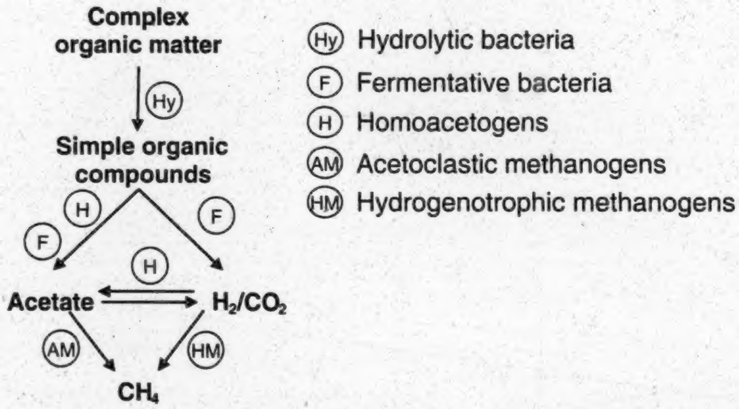
Complex organic matter
Hy
Simple organic compounds
H
F
F
H
Acetate
H₂/CO₂
AM
HM
CH₄
Hy Hydrolytic bacteria
F Fermentative bacteria
H Homoacetogens
AM Acetoclastic methanogens
HM Hydrogenotrophic methanogens

Figure 1-5: Schematic diagram of the degradation of organic matter in anaerobic environments. For simplicity, only acetate is shown as the product of fermentation. Adapted from Lay et al. (1998).

In the final conversion of acetate, CO_2 and H_2 to methane, many competing processes occur. Once all available electron acceptors such as oxygen, nitrate, sulfate, and oxidized iron species have been depleted in an environment, reduction of CO_2 with H_2 is the last available mode of energy generation for anaerobic microorganisms. This can occur in several different ways. The first is methane production by hydrogenotrophic methanogens, consuming CO_2 and H_2 and producing methane as a byproduct (Equation 1-1). The second is homoacetogenesis, which is the reduction of CO_2 with H_2 by homoacetogenic bacteria to produce acetate (Equation 1-2).

Equation 1-1: Hydrogenotrophic methanogenesis

$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ $\Delta G^{o\prime} = -135.6$ KJ/rxn*

Equation 1-2: Homoacetogenesis

$4H_2 + 2CO_2 \rightarrow CH_3COO^- + H^+ + 2H_2O$ $\Delta G^{o\prime} = -104.6$ KJ/rxn

*Free energy changes are given for standard conditions
(25°C, 1M substrates, pH 7, 1 atm pressure)

Another substrate available to anaerobic microorganisms in anaerobic environments is acetate, which is produced as a byproduct of the homoacetogens, or through fermentation of larger organic molecules by homoacetogens and other species of bacteria (Madigan et al., 2000). Acetate can be converted to methane and CO_2 by acetoclastic methanogens, according to Equation 1-3, although this produces much less energy than hydrogenotrophic methanogenesis (Madigan et al., 2000). Some homoacetogens have the ability to reverse the acetate-forming reaction, and can oxidize one molecule of acetate into two molecules of CO_2 and H_2 (Equation 1-4) (Schink, 1997). However, the oxidation of acetate is only thermodynamically favorable if a hydrogen scavenger, such as a hydrogenotrophic methanogen, is present and physically close to keep H_2 concentrations very low (Schink, 1997). In this case, the acetate-oxidizing bacterium is called a "syntroph," because of the obligate requirement for a syntrophic relationship with the hydrogen scavenger.

Equation 1-3: Acetoclastic methanogenesis

$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ $\Delta G^{o\prime} = -36$ KJ/rxn

Equation 1-4: Acetate oxidation

$CH_3COO^- + H^+ + 2H_2O \rightarrow 4H_2 + 2CO_2$ $\Delta G^{o\prime} = +104.6$ KJ/rxn

With all the possible interactions among microorganisms, anaerobic metabolism in the absence of inorganic electron acceptors can be very complex, and the dominant process in a given environment will depend on which mode of energy generation is most thermodynamically favorable at the given physical conditions, such as temperature, acetate concentration, hydrogen concentration, and the presence of the appropriate consortia of microorganisms (Lay et al., 1998).

The syntrophic oxidation of acetate is only favorable when it is coupled with hydrogenotrophic methanogenesis, by combining Equation 1-4 and Equation 1-1, providing each microorganism with half of the 31 kJ produced per reaction.

Equation 1-4: $CH_3COO^- + H^+ + 2H_2O \rightarrow 4H_2 + 2CO_2$ $\Delta G^{\circ\prime}$= +104.6 KJ/rxn).
Equation 1-1: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ $\Delta G^{\circ\prime}$= -135.6 KJ/rxn

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Equation 1-5: $CH_3COO^- + H^+ \rightarrow CH_4 + CO_2$ $\Delta G^{\circ\prime}$= -31 KJ/rxn

This process becomes more favorable at increasing temperature, and was first observed in thermophilic anaerobic digesters incubated at 58°C (Zinder and Koch, 1984). Syntrophic acetate degradation has since been observed in mesophilic cultures incubated at 37°C, but only when acetoclastic methanogens (which would otherwise compete for acetate with the syntrophic acetate-oxidizers) have been inhibited by other factors, such as high ammonium concentration (7 g NH_4^+-N /L)(Schnürer et al., 1994).

The first step in this syntrophic interaction, the hydrogen-producing fermentation of fatty acids, becomes less favorable as temperature decreases (Kotsyurbenko et al., 2004). This can be seen clearly in the degradation of acetate to CO_2 and H_2 (Equation 1-4). This reaction is endergonic under standard conditions (25°C, 1 M substrates, pH 7, 1 atm pressure), because the ΔG° and ΔH° for Equation 1-4 are both positive and unfavorable. However, the change in entropy, ΔS, is positive and favorable. Therefore, when looking at the equation $\Delta G^\circ = \Delta H^\circ - T\Delta S$, a positive ΔS makes ΔG° less endergonic, so when coupled with a hydrogen-consuming methanogen, this reaction can proceed.

At lower temperature, the degradation of acetate becomes less favorable. As the temperature decreases, the last term $T\Delta S$ becomes smaller, and therefore has a smaller effect on ΔG°. At a certain temperature threshold, approximately 15 to 20°C (Kotsyurbenko, 2005), this reaction is no longer favorable even when coupled with a hydrogen consuming methanogen. Thus, as temperature decreases, syntrophic fatty acid oxidation coupled with hydrogenotrophic methanogenesis becomes less favorable, and is out-competed by homoacetogenesis and acetoclastic methanogens.

In natural environments, all three types of microorganisms (homoacetogens, hydrogenotrophic methanogens and acetoclastic methanogens) will compete for available substrates. Physical and chemical factors such as temperature and acetate concentration will determine which microorganisms will dominate, depending on which will get the most energy from their form of metabolism. If the homoacetogens out-compete the hydrogenotrophic methanogens for H_2 and CO_2, the resulting acetate will be consumed by acetoclastic methanogens, to produce methane. It has been observed (Lay et al., 1998) that hydrogenotrophic methanogenesis and acetate production occur simultaneously in lake sediments, but with dominance of homoacetogenesis and acetoclastic methanogenesis. At lower temperatures, homoacetogens seem to be better able to compete with the hydrogenotrophic methanogens for H_2 and CO_2, and nearly all of the electron flow goes through acetate in low temperature lake sediment environments (Schink and Stams, 2002). Homoacetogens can also out-compete hydrogenotrophic methanogens because of their versatile metabolic properties, because they degrade organic matter to acetate in the absence of H_2 and CO_2 (Lay et al., 1998).

In the oil sands tailings environment, acetate is probably produced as a metabolite of primary and secondary fermentation, as well as from homoacetogenesis from H_2 and CO_2. Acetate, H_2 and CO_2 will subsequently be available as substrates for both the homoacetogens and the methanogens. Because homoacetogens produce more energy from H_2 and CO_2 at low temperatures than do hydrogenotrophic methanogens, homoacetogenesis will likely be the dominant process at temperatures below 20°C, which includes most of MLSB and WIP except the areas where tailings are being freshly deposited (at approximately 60°C). Acetoclastic methanogenesis can then proceed and convert the acetate to methane.

1.2.2 Microbiological Interactions in MLSB

Microbiological studies on MLSB MFT were done in 1985 by Foght et al. (1985). Aerobic and anaerobic heterotrophic microbes were detected, as well as SRB. The ability of microbes in the MFT to produce methane was also tested, because methane production was thought to hinder the densification process of the tailings. Methane could be produced from the MFT when they were amended with glucose and incubated at 37°C, but not when they were incubated at the *in situ* temperature of 15°C for 25 d (Foght et al., 1985). Therefore, methane production was not considered an important process occurring in MLSB.

Methane production in MLSB was first observed as bubbles of gas rising to the surface of the lake in the early 1990s. By 1996 methanogens had reached high enough numbers to be detected by the most probable number (MPN) method (Holowenko et al., 2000). The interactions between SRB and methanogenic populations in MLSB were studied by Holowenko et al. (2000). Methanogenic microorganisms were found at all depths sampled, down to 20 m. It was found that the sulfate concentrations decreased with depth, and therefore so did the numbers of SRB (Figure 1-6). The decrease in SRB may have allowed the methanogenic populations to increase, and the numbers of methanogenic microbes increased with depth as indicated by MPN analysis. However, at a depth of 5 to 8 m below the water surface, just below the water:tailings interface, there were high numbers of both methanogens and SRB (Holowenko et al., 2000). This observation suggests that there are high concentrations of nutrients at this depth, which can support high numbers of these competing populations.

Total methane yield produced from the tailings was measured from microcosms containing only MLSB tailings. Methane production accounted for less than 1% of the total dissolved and undissolved carbon present in the tailings after incubation for 516 days (Holowenko et al., 2000). Amendments with either acetate or H_2+CO_2 were both shown to stimulate methane production from the tailings, indicating that the methanogenic populations present can utilize either substrate, and suggest that the initial breakdown of residual hydrocarbons may be the rate-limiting step in methane production.

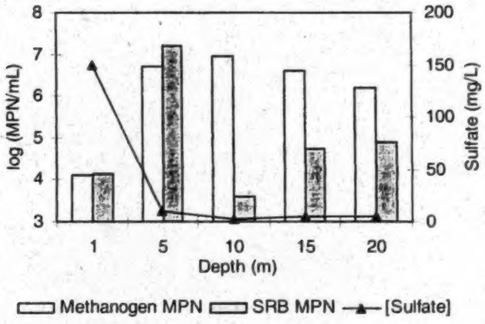


Figure 1-6: Depth profile of sulfate concentrations in MLSB as well as numbers of SRB and methanogens. Adapted from Holowenko et al. (2000).

1.2.2.1 Effects of Methane Production in MLSB

Methane production in MLSB was first thought to be detrimental to tailings consolidation and biological processes in the pond. The bubbles of methane rising up through the tailings were predicted to slow the densification process by resuspending fines and detrital material (Fedorak et al., 2002). Bubbles could also carry low molecular weight hydrocarbons and organic molecules (i.e. naphthenic acids) from the tailings into the water column which would be damaging to microorganisms in the water (Fedorak et al., 2002). Aerobic methylotrophic bacteria in the upper layers could oxidize the methane to CO_2, depleting the oxygen in the water and leading to anoxic conditions, which would again be damaging to aerobic microorganisms living in the water column (Fedorak et al., 2002).

Fedorak et al. (2003) performed column experiments intending to study the effects of the methane production on MFT densification. They found that in samples actively producing gas, the tailings densified fifteen times more rapidly than predicted from previous observations at MLSB (Figure 1-7). The addition of sulfate did not affect the amount of methane produced or the densification rate, and columns not producing gas did not show the same increased densification. These observations suggested that the increased rate of densification is a result of gas production, which agreed with on-site observations at MLSB that the tailings began to consolidate much more quickly following the onset of methane production (Fedorak et al., 2003).

The increase in densification is thought to be due to two processes. The first is the formation of drainage paths by the escaping methane bubbles, allowing the pore water to be released (Fedorak et al., 2003; Guo et al., 2004). The second mechanism is due to the production of CO_2 that accompanies methane production from acetate (Equation 1-3). The dissolution of CO_2 in water produces bicarbonate (HCO_3^-) and H^+, which decreases the pH of the fine tails and participates in cation exchange with divalent cations around the clay particles. This alters the stable gel structure of the fine tails and allows for more rapid densification (M. MacKinnon, personal communication).

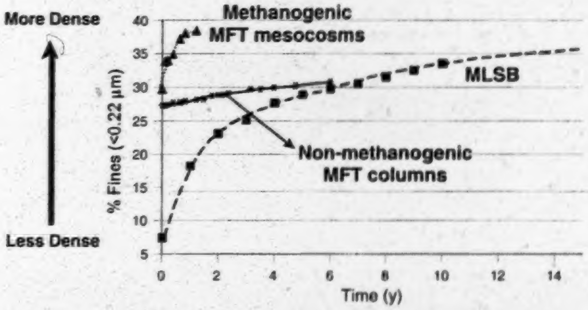


Figure 1-7: Densification over time of *in situ* MLSB tailings, predicted before methane production initiated in MLSB (squares), methanogenic MFT mesocosms (triangles) and non-methanogenic MFT columns (asterisk). Adapted from Fedorak et al. (2003).

1.2.2.2 Current research into physical processes of densification

The mechanism by which the gas production affects tailings densification is currently under study. In 2001, small columns filled with the tailings were monitored over time. It was found that at the end of the incubation period, columns amended with acetate and incubated at 25°C had a higher percent solids, lower water content and a higher gas-void ratio (all indicating these columns had a greater rate of densification) than unamended columns or those incubated at 6°C (Fedorak et al., 2003). Microbial activity, as indicated by gas production, appeared only to occur at ambient temperature and in the presence of an appropriate substrate. Microbially-active columns produced more methane, and even though more gas was trapped in the pore spaces, the solids content increased from 37.8% to 41.9%. The permeability of the tailings was also found to increase over time and this was thought to be the reason for the increased densification rate (R. J. Chalaturnyk, personal communication).

Ongoing column experiments conducted in the laboratory of R. J. Chalaturnyk (Guo et al., 2004) will hopefully reveal more information concerning the densification process. The effects of acetate amendment on microbial activity and gas generation were investigated, and how these processes affected the densification of the tailings. The tailings in the columns were allowed to initially consolidate at 4°C either under self-weight or applied pressure, and then were transferred to 25°C to initiate microbial gas production. Permeability, gas production, and interface height were all monitored at various times during the experiment, and samples were taken for chemical analysis and microbial enumeration. It was observed that acetate amendment greatly increased microbial activity and gas production, and therefore increased the densification rate of the tailings, by the two mechanisms described above.

1.2.3 Methanogens

The existence of microorganisms that produce methane was suggested as early as 1776, when Alessandro Volta discovered the production of "combustible air" released from sediments in streams, bogs and lakes (Balch et al., 1979). Methanogens were the first species of the domain Archaea to be identified, but they were first classified as species of Bacteria. However, in the 1970s, when the study of 16S ribosomal RNA (rRNA) gene sequences indicated that methanogens were in fact part of a lineage separate from the Bacteria, they were classified as part of the kingdom Euryarchaeota in the domain Archaea (Woese et al., 1990).

Methanogens are a unique group in the kingdom Euryarchaeota. They are the only known microorganisms that produce methane as a result of energy production. Some species of bacteria produce methane as a by-product of fermentative metabolism, but do not obtain any energy from this process (Jones et al., 1987). Methanogens are strict anaerobes, and can utilize only very simple carbon sources, such as formate, methanol, carbon monoxide, carbon dioxide, methylamines, dimethyl sulfide, methanethiol, and acetate (Ni and Boone, 1991; Lomans et al., 1999; Madigan et al., 2000). They exist in three main natural environments: anaerobic sediments, 1999; Madigan et al., 2000). They exist in three main natural environments: anaerobic sediments, the intestinal tracts of animals and insects, and chemolithotrophic environments. In nearly all anaerobic sediments and man-made anaerobic environments such as sewage digestors, methanogens carry out the final steps in the mineralization of organic matter (Jones et al., 1987; Garcia et al., 2000). Because methanogens can only grow on a narrow range of substrates, they require the presence of other microorganisms to break down complex organic substrates into simpler forms. In the intestinal tracts of ruminants, insects and many other higher organisms (including humans), diverse microorganisms produce metabolites such as volatile fatty acids including acetate. Some of these metabolites are absorbed and used for nutrients by the host animal, and some acetate is used by methanogens, also present in the intestinal tract, for the production of methane (Garcia et al., 2000). Finally, in environments with no organic carbon, such as hot springs and hydrothermal vents, methanogens are the primary producers and grow chemoautotrophically using H_2 as an energy source (Garcia et al., 2000).

The methanogens are taxonomically divided into five orders. The characteristics of selected genera in each order are summarized in Table 1-1. Methanobacteriales consists of nonmotile methanogens (Garcia et al., 2000) with morphologies ranging from cocci to long rods (Balch et al., 1979). Methanomicrobiales also have a range of morphologies, and use H_2+CO_2 to produce methane (Garcia et al., 2000). Methanococcales are irregular cocci, and use H_2+CO_2 and formate as substrates for methanogenesis (Garcia et al., 2000). The Methanosarcinales are all irregular cocci, and all use acetate for methane production. The most recently discovered order is Methanopyrales, which consists of only one species, *Methanopyrus kandleri*, isolated from both deep sea and shallow marine hydrothermal vents (Garcia et al., 2000). *M. kandleri* exist as rods in long chains (Balch et al., 1979), and have a type of pseudomurein in their cell envelope that is different from that of other methanogens (Garcia et al., 2000).

Methanogens can be very difficult to culture in the laboratory. They grow very slowly, and are easily out-competed by other microorganisms, so contamination is difficult to avoid. The growth requirements for methanogens existing in very chemically and physically complex environments such as the rumen of cattle (Garcia et al., 2000) are not completely understood.

Methanogens are also not very metabolically diverse and use a very limited range of substrates, which makes them difficult to identify based solely on metabolic characteristics (Wright and Pimm, 2003). For these reasons, methanogens are usually identified on a molecular basis, using 16S rRNA gene sequences (Garcia et al., 2000; Wright and Pimm, 2003).

Table 1-1: Characteristics of methanogenic Archaea [a]

		Substrates for Methanogenesis[b]							
Genus	**Morphology**	H_2+CO_2	For+CO_2	Pyr+CO_2	Alcohols	MeOH	Ac	$MeNH_2$	MeS
Methanobacteriales									
Methanobacterium	Long rods	•	•						
Methanobrevibacter	Short rods	•	•						
Methanococcales									
Methanococcus	Irregular cocci	•	•	•					
Methanomicrobiales									
Methanomicrobium	Short rods	•	•						
Methanospirillum	Spirilla	•	•						
Methanocorpusculum	Irregular cocci	•	•		•				
Methanoculleus	Irregular cocci	•	•		•				
Methanocalculus (genus insertae sedis) [c]	Irregular cocci	•	•						
Methanosarcinales									
Methanosarcina	Large irregular cocci in packets	•				•	•	•	
Methanolobus	Irregular cocci in aggregates					•		•	
Methanosaeta	Long rods to filaments						•		
Methanomethylovorans [d]	Large irregular cocci in aggregates					•		•	•
Methanopyrales									
Methanopyrus	Rods in chains, growth at 110°C	•							

[a] excerpted from Madigan et al. (2000) unless otherwise indicated
[b] Abbreviations used: For, formate; Pyr, pyruvate; MeOH, methanol; AC, acetate; $MeNH_2$, methylamines; MeS, methanethiol.
[c] (Garcia et al., 2001)
[d] (Lomans et al., 1999)

1.3 Using 16S rRNA genes to Describe Community Diversity

Microbial identification methods traditionally require the initial cultivation of the individual strains. Determining the exact culture conditions and substrates required by all possible microorganisms existing in an environment is impossible, so cultivation always introduces a bias in the species of microorganisms identified in an environment (Amann et al., 1995). Growth of a mixture of microorganisms selects for those that grow quickly and can efficiently use the substrate and conditions presented to them in the laboratory (Dunbar et al., 1997). This bias has been shown in studies of most environments, including marine, freshwater, activated sludge, soil and sediment: numbers indicated by microscopic total cell counts always exceed the viable cell counts by several orders of magnitude (Amann et al., 1995). Microbiologists awaited a technique which would allow them to identify microbes in an environment without cultivation.

rRNA sequences have attracted the most interest for use in the identification of uncultivable microbes. rRNA is very ancient molecule with extremely conserved structure, size and sequence. Small changes in sequence, and therefore secondary structure, of the rRNA molecule will indicate evolutionary distance. There has been no evidence of lateral 16S rRNA gene transfer between microorganisms, which means that sequence variations probably represent true evolutionary relationships (Pace et al., 1986). In prokaryotes, rRNA genes are arranged in operons (Figure 1-8). Microorganisms can contain anywhere from one to fifteen rRNA operons in their genome (Louws et al., 1999).

The 5S rRNA molecule is approximately 115 to 120 nucleotides long. Because the 5S rRNA sequence is so short, when 5S rRNA sequences are used in phylogenetic comparisons it is difficult to know if valid conclusions are being made. The 23S molecule is approximately 3000 nucleotides, so sequencing of the entire molecule is more difficult. Therefore, most focus has been on the 16S rRNA gene sequence, which is close to 1500 nucleotides long. The comparison of complete 16S rRNA gene sequences allows valid conclusions to be made for the phylogenetics of the microorganisms in question (Pace et al., 1986).

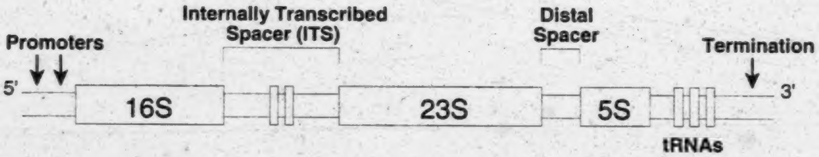


Figure 1-8: Arrangement of genes in the bacterial rRNA operon. Adapted from Louws et al. (1999).

16S rRNA gene sequences can be used in several ways to determine the phylogenetic placement of microbial species. DNA can be extracted from pure cultures of microorganisms and the 16S rRNA gene is isolated by the polymerase chain reaction (PCR) and sequenced. The 16S rRNA gene sequence can then be compared with those of previously isolated microorganisms to determine its phylogenetic placement. Alternatively, total community DNA can be extracted from an environmental sample and the mixture of 16S rRNA gene sequences can be cloned and analyzed. Each clone can be directly sequenced to determine the species of microbes present in the environment (Weisburg et al., 1991). This makes it unnecessary to culture all of the microorganisms present in an environment, and provides a less biased look at community diversity. In environments with high diversity, many clones must be sequenced to obtain an accurate representation of the diversity present. Sequencing is often expensive, laborious and requires specialized equipment. This can restrict the number of clones analyzed, thus restricting how completely an environment is described.

1.3.1 Amplified Ribosomal DNA Restriction Analysis

Amplified Ribosomal DNA Restriction Analysis (ARDRA) is a modification of the direct sequencing of 16S rRNA gene clones, by first screening the clones using restriction analysis (Figure 1-9). Genomic DNA is extracted from the environmental sample, and 16S rRNA genes are amplified by PCR from the genomic DNA. The amplified sequences are cloned into an appropriate plasmid vector for construction of a clone library in *Escherichia coli*. Instead of directly sequencing the clones, the 16S rRNA gene fragments are reamplified and digested using a tetrameric restriction endonuclease, such as *Hae*III (5'-GG/CC-3') or *Cfo*I (5'-CGC/G-3') (Martínez-Murcia et al., 1995). Separation of fragments on an agarose gel generates a pattern of bands, a "fingerprint," depending on slight species-specific sequence variations of the 16S rRNA gene sequence. Each unique fingerprint represents an operational taxonomic unit (OTU) or ribotype. Two clones which generate the same fingerprint are assumed to have the same 16S rRNA gene sequence and therefore represent the same microorganism or very closely related microorganisms (Martínez-Murcia et al., 1995).

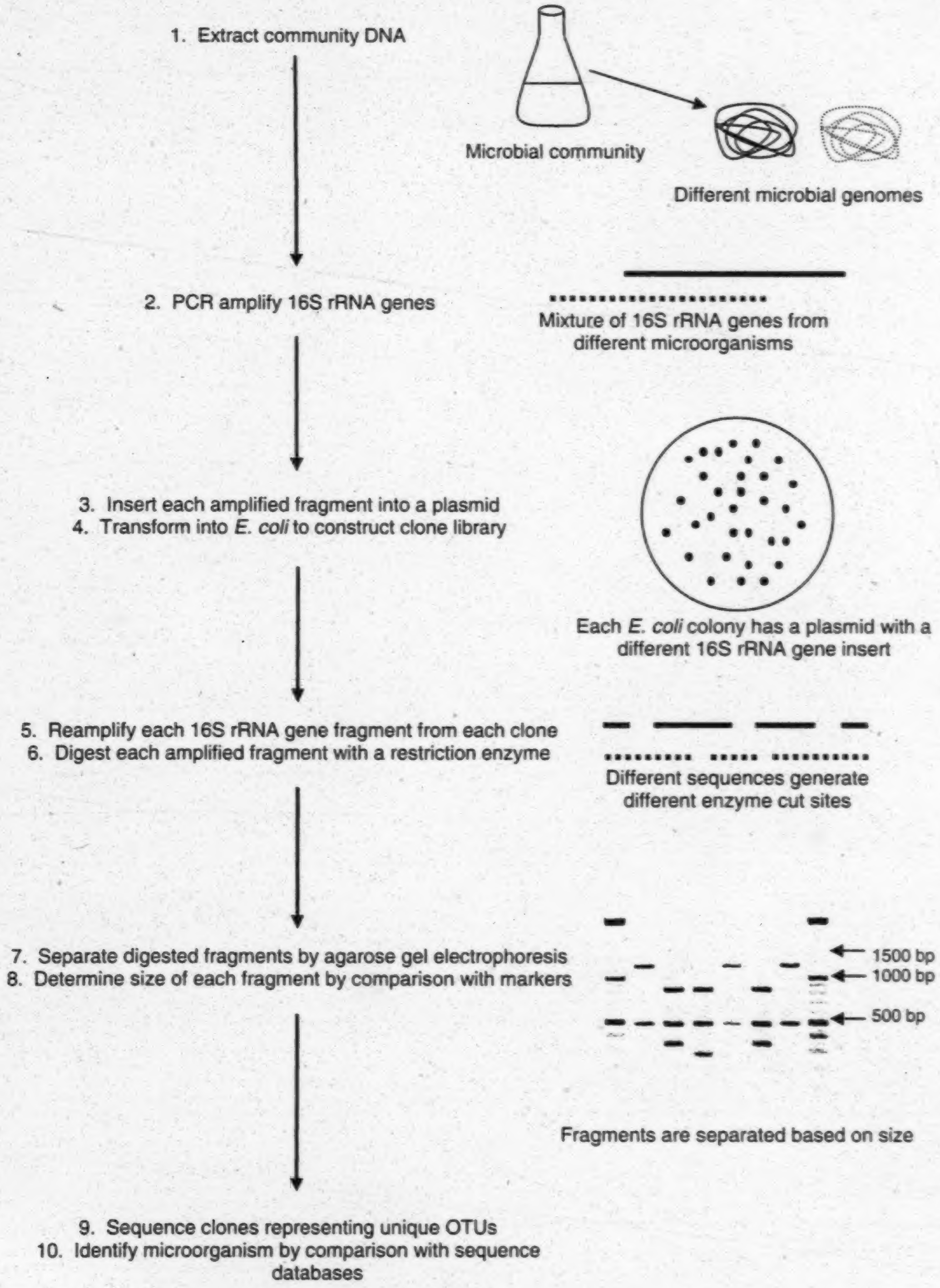

1. Extract community DNA
Microbial community
Different microbial genomes
2. PCR amplify 16S rRNA genes
Mixture of 16S rRNA genes from different microorganisms
3. Insert each amplified fragment into a plasmid
4. Transform into E. coli to construct clone library
Each E. coli colony has a plasmid with a different 16S rRNA gene insert
5. Reamplify each 16S rRNA gene fragment from each clone
6. Digest each amplified fragment with a restriction enzyme
Different sequences generate different enzyme cut sites
7. Separate digested fragments by agarose gel electrophoresis
8. Determine size of each fragment by comparison with markers
1500 bp
1000 bp
500 bp
Fragments are separated based on size
9. Sequence clones representing unique OTUs
10. Identify microorganism by comparison with sequence databases

Figure 1-9: Method of identification of microorganisms using ARDRA.

One (or more) representative clone from each phylotype is then selected for sequencing to identify the ribotype. This technique permits microbial communities to be analyzed without the need for cultivation, and therefore can detect previously uncultured or "uncultivable" microorganisms (Martínez-Murcia et al., 1995). ARDRA also greatly decreases the number of clones to be sequenced, as only one representative from each OTU needs to be sequenced (Moyer et al., 1996).

The restriction endonucleases used for the screening must be chosen carefully. If the fragments generated by digestion are too large, variation could be missed within the large fragments. Conversely, if 16S rRNA sequences of two different microorganisms are very similar, one restriction enzyme may not be able to distinguish between different microorganisms. Ideally, several restriction enzymes should be tested to find the optimal number and type to generate patterns accurately reflecting the diversity in an environment (Engebreston and Moyer, 2003). Wright and Pimm (2003) identified the best restriction endonucleases for the analysis of methanogenic species. They determined that digestion with *Hae*III could generate distinct patterns to differentiate between 15 of the 26 strains studied. Additional differentiation could be achieved by digestion with *Alu*I, *Hpa*II, *Fok*I, and *Mlu*NI (Wright and Pimm, 2003).

Another use for the "fingerprints" generated by ARDRA analysis is for direct determination of evolutionary relationships without sequencing (Heyndrickx et al., 1996; Moyer et al., 1996). Restriction fragment lengths are subjected to numerical analysis as described by Nei and Li (1979) to calculate evolutionary distances. The groupings based on ARDRA patterns from known microorganisms predict the same evolutionary relationships as when the entire sequences themselves are aligned (Heyndrickx et al., 1996). This indicates that numerical analysis of ARDRA patterns is a valid method of determining phylogenetic relationships.

The "coverage" of clone libraries describes how well the library represents the true diversity of DNA sequences in the original sample. It can be estimated using several methods, allowing one to determine if the size of the clone library is sufficient to describe the diversity of microorganisms in the environment under study (Kemp and Aller, 2004). The oldest, and most common method, is Good's Coverage (C), defined by the equation:

Equation 1-6: $C = 1 - (n_1 / N)$

where C is coverage, n_1 is the number of OTUs that appear only once in the clone library and N is the total number of clones in the library (Good, 1953). Another method of calculating coverage considers the number of "rare" OTUs, instead of the total number (Kemp and Aller, 2004).

Equation 1-7: $C_{ACE} = 1 - (F_1/N_{rare})$

where F_1 is the number of OTUs that appear only once, and N_{rare} is the total number of clones in OTUs occurring 2 to 10 times. This method generally provides a lower value for coverage than Good's coverage (Kemp and Aller, 2004). More than one definition of coverage should be used when evaluating clone library size (Kemp and Aller, 2004).

Another way to estimate if sufficient clones have been analyzed to accurately describe the diversity present in an environment is through an accumulation curve, plotting the number of OTUs found *versus* number of clones sampled. If an environment has been exhaustively sampled, this should result in an asymptotic curve (Kemp and Aller, 2004). Two examples of accumulation curves are shown in Figure 1-10, the first illustrating a case where the environment has been exhaustively sampled, and the second illustrating the case where it has not.

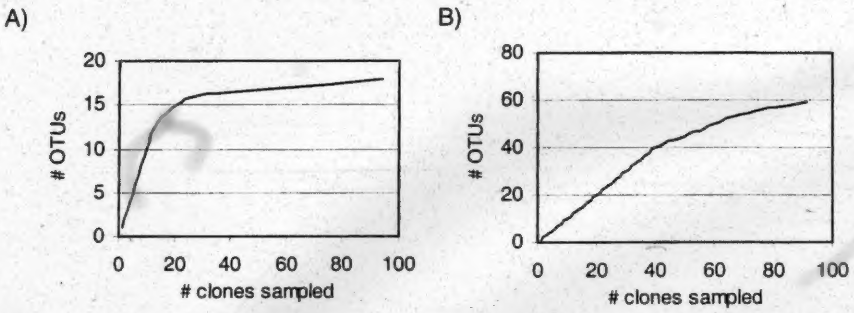


Figure 1-10: Examples of accumulation curves constructed from clone libraries which A) have and B) have not exhaustively sampled the diversity present in an environment.

Estimators of species richness are also useful for defining the diversity of microorganisms in an environment based on 16S rRNA clone library analysis. A common estimator of species is the Shannon-Weaver index (Shannon and Weaver, 1949), which is defined by the equation:

Equation 1-8: $$H' = -\sum_{i=1}^{D} a_i \ln a_i$$

where H' is the Shannon-Weaver index, D is the number of OTUs *i*, and a_i is the proportional abundance of each OTU *i*. The Shannon-Weaver index can be used to compare the species richness of different environments by comparing the clone library data from those environments.

Caution must be used when making conclusions regarding the abundance of each species living in an environment using ARDRA. The frequency of a clone in the clone library may not necessarily reflect the frequency of that microorganism in the environment due to biases associated with each step involved in the clone library construction and screening. Two of the steps that cause the most bias in community analysis by ARDRA are the extraction and purification of DNA from the environmental sample and the PCR amplification of the 16S rRNA genes from the total community DNA. These two steps are outlined in further detail below.

Another potential source of misinterpretation in the ARDRA technique is that only detects DNA sequences are detected, not viable microorganisms. Microorganisms represented by the sequences detected may be present but not actively growing in the environment, or are dead and their DNA has not yet degraded. Sequences that are detected in lower proportions than others must be interpreted carefully, as they may not be an important part of the active microbial community. This is especially important when trying to determine the metabolic roles of microorganisms present in an environment.

1.3.2 DNA Extraction and Purification

DNA extraction is the first step in any molecular analysis of a microbial community. Environmental samples usually contain a complex mixture of microorganisms and organic molecules. Many methods have been developed for the extraction of genomic DNA from microorganisms. Chemical lysis involves using chemicals such as guanidium isothiocyanate, hexadecyltrimethyl-ammonium bromide (CTAB), or detergents such as sodium dodecyl sulfate (SDS), sometimes in the presence of heat (Roose-Amsaleg et al., 2001). Enzymes such as proteinase K, pronase or lysozyme can also be used to disrupt the cell walls of bacteria to release high molecular weight DNA (Roose-Amsaleg et al., 2001). Mechanical methods such as bead-beating, freeze-thaw cycles (Tsai and Olson, 1991), microwave heating or sonication are sometimes employed as a less selective method of DNA extraction resulting in sheared DNA, and are often useful for species that are difficult to lyse using the other methods (Roose-Amsaleg et al., 2001). Often a combination of two or more methods is used to ensure optimal cell lysis.

Bead beating and bead-mill homogenization are two of the most common techniques for direct DNA extraction from environmental samples. They are simple, relatively high throughput, and allow for very efficient lysis of cells, especially when combined with SDS (Moré et al., 1994). However, these techniques are biased in that large cells are lysed more efficiently than small cells. Moré et al. (1994) found that about 6% of cells were not lysed by bead-mill homogenization, determined by direct observation with laser scanning epifluorescence microscopy. Most of these cells were small cocci, indicating that they were able to survive treatment with a bead-mill homogenizer. Bead beating can also lead to shearing of the DNA, and short pieces of DNA can cause formation of chimeric sequences during PCR (Zhou et al., 1996; Roose-Amsaleg et al., 2001). The method of DNA extraction must be chosen to achieve a balance between unbiased lysis of all microorganisms present, and prevention of shearing of DNA (von Wintzingerode et al., 1997; Roose-Amsaleg et al., 2001).

After cell lysis, DNA must be separated from the rest of the cellular components, such as proteins, polysaccharides, and RNA. There are many methods to accomplish this, and the most common are precipitation with phenol-chloroform, phenol-isoamyl alcohol, ammonium acetate, or NaCl (Roose-Amsaleg et al., 2001). After the cellular components have been removed by precipitation and centrifugation, the DNA is precipitated with ethanol or isopropanol. Polyethylene glycol can also be used to precipitate DNA; however this can interfere with subsequent PCR amplification. Phenol extraction is required to remove the polyethylene glycol, which greatly reduces the yield of DNA (Roose-Amsaleg et al., 2001).

Humic and fulvic acids are contaminants often present in DNA extracted from soil environments and can interfere with restriction digestion or the PCR reaction by binding to the enzymes involved (von Wintzingerode et al., 1997; Cullen and Hirsch, 1998). The phenolic groups in humic acids bind to the amide group of the polypeptide, or are oxidized to quinones and then bind to DNA or proteins (Wilson, 1997).

The DNA purification step is critical for DNA extraction from soils, and methods have been developed to eliminate humics from the DNA. Polyvinylpolypyrrolidone (PVPP) can be used to remove the humic acid contamination from the DNA before PCR amplification (Young et al., 1993; Cullen and Hirsch, 1998). Passing the DNA through a polyacrylamide gel (Tsai and Olson, 1992) or an agarose gel containing PVPP allows the humic acids to migrate at a different speed than the DNA (Cullen and Hirsch, 1998) and therefore be separated. Subsequent gel purification produces pure genomic DNA. Organic inhibitors can also be removed by treating the extracted DNA with surfactants or chelating agents, such as Triton X-100 or ethylene diamine tetraacetic acid (EDTA), prior to cell lysis (Fortin et al., 2004). Optimizing the DNA purification step is more important than optimizing DNA yield, because increased yield does not necessarily mean the diversity of the environment is represented (Stach et al., 2001).

1.3.3 PCR Amplification and Bias

Total community DNA extracted from an environmental sample is subsequently used for amplification of the 16S rRNA gene by PCR. PCR amplification of 16S rRNA genes from genomic DNA is a simple way to obtain a mixture of 16S rRNA gene fragments, hopefully representing the diversity of 16S rRNA genes in the environmental sample.

The choice of primers is the first step in determining the range of microorganisms that will be amplified by the PCR reaction (von Wintzingerode et al., 1997). Primers are usually designed based on the sequences of previously isolated and characterized microorganisms, obtained from public databases. Primers can be designed to be group- or domain-specific, or can be "universal" and theoretically capable of amplifying 16S rRNA gene sequences from all prokaryotic species (Baker et al., 2003). Primers are designed to be complementary to conserved regions of the 16S rRNA gene, but these sequences are not exactly conserved across all existing species (Baker et al., 2003). For this reason, degeneracies can be introduced into the primer sequence to allow it to anneal to more than one template sequence with minimal mispairing of bases.

There are many sources of bias associated with the PCR amplification step, which are explained in further detail below.

1.3.3.1 PCR Inhibitors

Organic compounds coextracted with DNA from the environmental sample can inhibit PCR by binding to *Taq* polymerase. The effects of PCR inhibitors can be reduced by simply diluting the sample, which dilutes the inhibitor but also dilutes the template DNA (Roose-Amsaleg et al., 2001). This is not a favorable situation because any rare sequences present in the environment may not be amplified and subsequently not represented in the clone library (von Wintzingerode et al., 1997; Stach et al., 2001). Sequestrating agents such as bovine serum albumen (BSA) or T4 gene 32 protein can also be added to interact with the inhibitors and prevent them from interfering with the function of the *Taq* polymerase (Roose-Amsaleg et al., 2001). DNA purification steps must be optimized to ensure complete removal of PCR inhibitors.

1.3.3.2 Variations in rRNA Gene Copy Number

Microorganisms may contain anywhere from one copy of the rRNA operon (mycoplasma) to 15 (*Clostridium paradoxium*) (von Wintzingerode et al., 1997; Louws et al., 1999). A high rRNA gene copy number has been correlated with the ability of a microorganism to respond quickly to changes in its environment, such as nutrient addition (Klappenbach et al., 2000). More copies of rRNA genes means more ribosomes will be present in these cells, and therefore the cell will be able to synthesize catabolic or stress response proteins quickly when required. If the adaptable microorganisms contain more copies of the rRNA operon, they will likely be over-represented in the clone library (Farrelly et al., 1995; Crosby and Criddle, 2003). This may result in the same bias as culturing, because enrichment cultures or plating selects for the fast-growing microorganisms which respond quickly to new conditions presented to them in the laboratory. The sequences of the genes are not necessarily identical in each copy of the operon, and this will cause the overestimation of community diversity when 16S rRNA genes are amplified from an unknown mixture in an environmental sample. It has been shown that 16S rRNA genes from the same microorganism can have more than a 5% sequence variation (Mylvaganam and Dennis, 1992; Amann et al., 2000).

1.3.3.3 Preferential Amplification

Certain gene sequences are preferentially amplified by the PCR reaction, resulting from several factors. The mole %GC content of the genome of a microorganism is the most important factor influencing preferential amplification (von Wintzingerode et al., 1997). Because high %GC DNA has more hydrogen bonds holding the two strands together, these molecules will separate more slowly during the denaturation step of the PCR, and therefore will be less available for primer annealing and elongation. Low %GC DNA will bind primers more frequently and therefore will be amplified in a greater amount (Reysenbach et al., 1992) causing overrepresentation of these sequences in the clone library (Suzuki and Giovannoni, 1996). The addition of a denaturant such as acetamide, dimethyl sulfoxide (DMSO), or β-mercaptoethanol can decrease the melting temperature of the DNA and facilitate strand separation, allowing equal amplification of high and low %GC templates (Reysenbach et al., 1992).

Another cause of preferential amplification is due to differences in primer binding energies, and is a problem especially when degenerate primers are used (Suzuki and Giovannoni, 1996). 16S rRNA genes with high %GC primer binding sites favor binding of certain primers in the degenerate mixture which have the most G or C bases, because of the stronger hydrogen bonding of G-C pairs compared with A-T pairs (Polz and Cavanaugh, 1998). Therefore these genes will be amplified more frequently than genes with low %GC primer binding sites.

A potential problem with universal primers is that they may not recognize uncultured microorganisms with sequences that are significantly different from previously detected microorganisms. This phenomenon was demonstrated by Huber et al. (2002) after the discovery of a new species of Archaea, "*Nanoarchaeum equitans*" by microscopic means. They found that this microorganism did not stain with fluorescence *in situ* hybridization (FISH) when probes toward the 16S rRNA gene of Crenarchaeota and Euryarchaeota were used. However, when the 16S rRNA gene of "*Nanoarchaeum equitans*" was sequenced and probes designed specifically for it, the microorganism could be stained by FISH (Huber et al., 2002). This finding shows that the sequence of the primers or probe used can cause a bias against the detection of some novel microorganisms.

The formation of secondary structures by the single stranded template DNA can prevent binding of the primers, or can cause the *Taq* polymerase to pause or fall off the template DNA resulting in termination of elongation (Suzuki and Giovannoni, 1996). The formation of secondary structure in the flanking regions around the 16S rRNA gene can affect the ability of primers to anneal to these regions (Hansen et al., 1998). Secondary structure formation can be reduced by the addition of a denaturant such as acetamide (Suzuki and Giovannoni, 1996), or using different sets of primers that anneal to different locations of the gene (Hansen et al., 1998).

Total community DNA contains a mixture of 16S rRNA genes with slightly different sequences in various relative amounts. However, the proportion of each gene before amplification may not be reflected in the proportions of amplified gene products. Experiments by Suzuki and Giovannoni (1996) found that two different sequences in multi-template PCR tended toward a 1:1 ratio independent of what the initial template ratios were. They proposed that template sequences present in a higher initial concentration reach a maximal concentration and plateau more quickly, and this allows the sequences present in lesser amounts to "catch up" (Suzuki and Giovannoni, 1996). This source of bias can be reduced by simply reducing the number of cycles in the PCR reaction to the minimal number required (Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997).

1.3.3.4 PCR Drift

PCR drift is an effect that is distinct from preferential amplification. Preferential amplification is a reproducible tendency of a PCR reaction to amplify certain gene sequences over others in a multi-template mixture. However PCR drift is not reproducible because it is caused during the initial cycles of the PCR reaction, due to the different accessibilities of certain sequences over others in the mixture of genomic DNA templates (Polz and Cavanaugh, 1998). If a certain sequence is more accessible in the mixture of genomic DNA, it will be amplified to a greater extent during the first cycles, and therefore there will be more of that template available for amplification in later cycles. PCR drift can be reduced by performing several PCR amplifications of the same template DNA simultaneously, and then pooling the amplification products for use in cloning and restriction analysis (Polz and Cavanaugh, 1998).

1.3.3.5 Chimera formation

Chimeric sequences are formed when an incompletely extended molecule anneals to a similar sequence in the PCR reaction mixture (Meyerhans et al., 1990; Liesack et al., 1991). Incomplete elongation can be caused by *Taq* polymerase pausing on the gene and not having time to extend it completely, or premature termination due to formation of a secondary structure by the single stranded DNA (Meyerhans et al., 1990). The use of low molecular weight, or "sheared" DNA in the PCR reaction increases the chances that chimeric molecules will be formed (Liesack et al., 1991; Zhou et al., 1996). Fragmented 16S rRNA genes will produce incomplete amplification products, which can then anneal to similar sequences and form chimeras.

The formation of chimeras in the process of phylogenetic analysis of microbial communities in environmental samples can lead to the overestimation of biodiversity in the environment, because the chimeric molecules will form their own ARDRA pattern in restriction analysis, or a separate band in DGGE analysis (Kopczynski et al., 1994). The entry of chimeric sequences into public databases reduces the quality of the databases, because these sequences suggest the presence of a microorganism that does not exist (Kopczynski et al., 1994).

Chimeras can be identified by examining the secondary structure of the 16S rRNA sequence and determining if there are mismatches that could be formed by the fusion of two different sequences (Liesack et al., 1991). However, analysis of secondary structure does not always detect chimeric sequences, especially if the two combined sequences are closely related (Kopczynski et al., 1994). Phylogenetic comparisons of the 5' and 3' ends of the sequence separately can indicate the presence of a chimera if each end of the gene falls into a different phylogenetic clade (Kopczynski et al., 1994). More recently, computer programs have been developed, such as CHIMERA_CHECK (Ribosomal Database Project II [http://rdp.cme.msu.edu/html]), which compare a new sequence with those already present in the database and determine if it is a likely chimera (Hugenholtz and Huber, 2003).

A study by Hugenholtz and Huber (2003) demonstrated the seriousness of chimeric sequences. *In silico* analysis of nine published and three unpublished studies revealed 21 interphylum and 18 intraphylum chimeric sequences, and it was predicted that even more chimeras could have formed between very closely related sequences (Hugenholtz and Huber, 2003). It is difficult to identify chimeric sequences once they have been entered into the public databases, because programs such as CHIMERA_CHECK only compare new sequences with those already entered, but cannot compare sequences *within* the database (Hugenholtz and Huber, 2003).

1.3.3.6 Misincorporation

Point mutations can be formed during the PCR amplification, due to the low fidelity of most commercial polymerases. It has been calculated that the rate of misincorporation is about 5 mistakes per gene per PCR amplification (von Wintzingerode et al., 1997). This is a very low rate, a less than 0.3% change over the entire 16S rRNA gene sequence, and does not have a significant effect on the final phylogenetic analysis. However, if more than one amplification is to be done, such as in nested PCR (when an initial amplification with universal primers is followed by amplification with group-specific primers), the misincorporation rate will be additive and could pose a problem with phylogenetic comparisons (von Wintzingerode et al., 1997). Polymerase errors have also been shown to increase when the template is a mixture of homologous genes of slightly different sequences (Speksnijder et al., 2001). Genes may have "mutational hot-spots" which have a higher frequency of mutation than other locations in the gene (Qiu et al., 2001). Misincorporation can simulate increased diversity if new restriction patterns are generated in ARDRA analysis, by creating an artificial restriction site or abolishing a site that would normally be present (Qiu et al., 2001).

1.3.3.7 Contamination

Contaminating DNA can be introduced during sampling, through reagent solutions, reaction tubes, or airborne particles (von Wintzingerode et al., 1997). The 16S rRNA genes from the *E. coli* host into which the environmental genes are cloned could be amplified if 16S rRNA-specific primers are used for reamplification; therefore plasmid-specific primers should be used for reamplification of the 16S rRNA genes for restriction analysis (von Wintzingerode et al., 1997). Contamination creates artificial community diversity, as sequences detected are not present in the original environment but are introduced during sample processing. A laboratory performing PCR amplification using 16S rRNA gene-specific primers should have a set of micropipetters and reagents dedicated solely for preparation of PCR reactions. Only filtered tips should be used, and tips and tubes may be decontaminated by UV irradiation before use. Negative controls containing only reagents should be included in each DNA extraction and PCR amplification to ensure the absence of contamination (von Wintzingerode et al., 1997).

1.3.3.8 Cloning Bias

A last source of bias, although not caused during the PCR reaction but following it, is the cloning system used to separate the mixture of amplified 16S rRNA genes into plasmids for transformation into an *E. coli* host. Different cloning systems can include blunt end cloning, sticky end cloning, and TA cloning. Blunt end cloning has a very low efficiency, so not all 16S rRNA gene sequences will be cloned into a plasmid vector. Sticky end cloning involves digesting the ends of the amplified 16S rRNA gene sequence with restriction enzymes, and may result in cutting inside the gene itself, preventing cloning of the complete gene. TA cloning takes advantage of the fact that *Taq* polymerase leaves overhanging "A" bases on the ends of the amplified sequences, so the sequences can be cloned into vectors with overhanging "T" bases, therefore increasing the efficiency of blunt end cloning and preventing digestion within the 16S rRNA gene sequence. However, due to unknown factors, different sequences of DNA may have different cloning efficiencies, causing cloning bias with any cloning system used (Rainey et al., 1994).

1.3.4 Precautions to Prevent Misrepresentation of Diversity

Phylogenetic comparisons of 16S rRNA gene sequences obtained from clone libraries sometimes results in "bushes" of related clones on phylogenetic trees. These closely related sequences may represent the true diversity in an environment, but may also be due to the effects of procedural biases and PCR artifacts (Speksnijder et al., 2001). It is therefore good practice to group sequences that are greater than 97% similar as representing similar species (Speksnijder et al., 2001). Species may be excluded from detection by ARDRA if the cells are not lysed during DNA extraction, if the 16S rRNA gene sequence is unable to be amplified during PCR, or if sequence variation is missed by using an inappropriate restriction enzyme during screening by restriction analysis.

To avoid misrepresentation of diversity during ARDRA, several recommendations can be made:

1. Use a DNA extraction method which produces the highest molecular weight DNA possible. This will prevent the formation of chimeric sequences during PCR (Liesack et al., 1991; von Wintzingerode et al., 1997).
2. Avoid designing primers to contain degeneracies, and if this is not possible, use more than one primer set and mix the amplification products (Polz and Cavanaugh, 1998). Also, it is a good idea to perform more than one PCR reaction using the same genomic template and primers, and pool the reactions to use in cloning. Decreasing the annealing temperature can decrease the bias caused by different binding energies of primers, but this may cause non-specific amplification (Ishii and Fukui, 2001).
3. Use the fewest cycles possible in the PCR reaction, because any PCR bias present will be multiplied with each amplification cycle, and sequences will tend toward a 1:1 ratio (Suzuki and Giovannoni, 1996). Enough PCR product can usually be obtained from five to ten cycles, especially if several reactions are pooled. Chimeric sequences can also be reduced by decreasing the number of cycles (Wang and Wang, 1997).
4. Add a denaturant to the PCR reaction mixture to prevent the formation of secondary structures in the template and facilitate the separation of high %GC strands (Reysenbach et al., 1992; Suzuki and Giovannoni, 1996; von Wintzingerode et al., 1997).
5. Use a high template concentration for PCR amplification, because dilution of the template may prevent rare sequences from being amplified and included in the clone library (von Wintzingerode et al., 1997; Polz and Cavanaugh, 1998; Stach et al., 2001). This may

require optimization of DNA purification if DNA is being extracted from soil environments containing high concentration of humic acids and other inhibitors (von Wintzingerode et al., 1997; Cullen and Hirsch, 1998).

6. Choose a frequently cutting restriction enzyme so a large number of patterns are generated when screening the clone library. More than one enzyme may be required to generate additional distinguishing patterns (Moyer et al., 1996). This will provide a better chance that closely related species will be distinguished by the restriction analysis.
7. Strict aseptic technique and use of dedicated micropipettors should be used for DNA extraction and PCR amplification steps to ensure that foreign DNA is not introduced. Negative controls containing only reagents should be processed along with the samples to ensure there is no contaminating DNA (von Wintzingerode et al., 1997).
8. Be cautions in interpreting sequences that occur in low proportions, as these may represent microorganisms which are not active in the community.

Compared with biases introduced by culture-based identification methods, molecular techniques pose an enormous advantage for the analysis of environmental microbial communities. If proper precautions are taken to optimize each step and reduce bias as much as possible, ARDRA can be a very useful technique to obtain a qualitative picture of the species living in an environment. Caution must be taken when inferring quantitative conclusions from the data obtained from clone libraries, but ARDRA and related techniques are providing a broader view of microbial diversity on Earth. Culturing is still a valid microbiological technique, because it allows the determination of phenotypes of the microorganisms present, and provides information about what biochemical roles the microorganisms are playing in the environment (Grosskopf et al., 1998). Culturing can also be used in conjunction with molecular methods to first enrich for a specific metabolic group of microorganisms and decrease the total diversity to be analyzed. For example, if the goal of a study is to determine methanogenic diversity, then the use of methanogenic media will select for methanogenic organisms, and eliminate many of the other species that may not be involved in methane production. Employing both culture-based and molecular methods for study of microbial communities is therefore useful.

1.4 Thesis Objectives

Oil sands tailings management is becoming an increasingly prominent challenge for the oil sands companies. Much effort is placed on finding effective and economically feasible methods to manage the enormous volumes of tailings produced. Methane production is involved in the increased consolidation rates of the oil sands tailings, and there is more to learn about this process and how it can be used to the advantage of the oil sands companies.

The goal of this thesis was to identify the major methanogenic species present in MLSB and the bacterial species that support them. Eventually, this information could be used to stimulate these important species in the MFT and hopefully increase the densification rate further. It may also be possible to determine the source of the methanogenic communities and provide a methanogenic "inoculum" to oil sands companies whose tailings ponds have not yet become methanogenic. Because the cultivation of methanogens is very difficult, molecular methods, specifically ARDRA, were employed to study the methanogenic community composition in MLSB.

The main objectives of this thesis were:

1. To identify the dominant species of methanogens present in the tailings. The first experiments involved acetate-amended serum bottle microcosms inoculated with MFT obtained from MLSB. Samples from the serum bottle microcosms were used to inoculate MPN enumeration tubes, and these culture tubes were used for extraction of genomic DNA and construction of 16S rRNA gene clone libraries. This allowed the optimization of the clone library technique, and showed that methanogens could be identified in the methanogenic cultures. The reason for using the MPN culture tubes was to circumvent many of the contaminants (clay particles and bitumen) from the tailings which may interfere with molecular methods.
2. To identify methanogenic and bacterial species in cultured MFT and to study the effects of acetate amendment on the microbial populations. This research was done in conjunction with column studies attempting to determine the mechanics of how gas production increases the densification rate of the tailings (Guo et al., 2004). Samples were obtained from acetate-amended or unamended columns before and after incubation

and were used in enumeration of methanogenic microorganisms using the MPN method. The changes in the Archaeal (methanogenic) and Bacterial populations in the MPN cultured tubes were monitored over time and treatment type using ARDRA. Again, DNA was extracted from MPN culture tubes to circumvent problems with contaminants from the MFT.

3. To optimize DNA extraction from the neat, uncultured MFT, taken directly from the settling basins. Modified extraction procedures were tested to determine if enough DNA is present in the MFT, and if contaminants such as clay particles and residual bitumen can be removed.
4. To analyze microbial species present in the *in situ* MFT. Samples were obtained from various depths of MLSB and WIP, and the DNA extracted from the neat (uncultured, unmodified) tailings was used to construct Bacterial and Archaeal 16S rRNA clone libraries as described. The identification of species present at different depths (representing different tailings age) of the tailings pond provided a clearer picture of the microbial interactions occurring in the tailings pond.
5. To attempt to extract DNA from the oil sands ores. Samples were taken from the Syncrude oil sands ore. MPN enumeration of SRB and methanogens in the oil sands ore was also done. If it had been possible to obtain DNA from the ores, it would have been used to determine if the same methanogenic species are present in the MFT as in the original oil sands.

The cumulative results from this project were used to propose a pathway for methane generation in the tailings ponds and the cultured MFT. An amendment strategy was also proposed to potentially enhance methane generation in the *in situ* MFT, which will eventually increase densification rates. The questions raised by the results of this project allowed future experiments to be designed, providing further understanding of the microbial interactions in oil sands tailings.

2 Materials and Methods

2.1 Controls and Precautions

To prevent PCR bias and misrepresentation of diversity, several precautions were taken as discussed above (Section 1.3.4). Aseptic technique was used with all samples and reagents, using a dedicated set of micropipetters and bench space. Reagents were prepared with aseptic technique using sterile Milli-Q water and chemicals reserved solely for PCR applications and sterilized either by passing through a 0.22 μm filter or by autoclaving. Negative controls were included in every DNA extraction and PCR amplification step using the Archaeal- or Bacterial-specific primers. Degenerate primers were used that have been shown to amplify the 16S rRNA genes of a range of Archaeal species (DeLong, 1992) and Bacterial species (Saul et al., 2005) to ensure accurate representation of species present in the samples. Duplicate DNA extractions were performed in case the microbes were not homogeneously distributed in the samples. Three replicate PCR amplification reactions were performed simultaneously on each replicate DNA extraction, and the reactions were pooled to construct the clone libraries. Pooling the PCR reactions also allowed the number of cycles in the PCR amplification to be kept to a minimum.

2.2 Samples analyzed

2.2.1 Preliminary Microcosm Studies

MFT samples were taken in 2003 from MLSB. Methanogenic microcosms containing 100 mL MFT and 5 mL tailings pond water were amended with 1.5 g acetate /L and incubated in the dark at room temperature. The three tube-MPN method (See Section 2.7.1) was used to enumerate methanogenic populations when the methane concentration in the headspace reached 0, 10, 20, 30 and 40% (v/v) methane. This work was performed by Jessica Hrudey.

Optimization of the PCR amplification of Archaeal 16S rRNA gene fragments, DNA extraction from the MPN culture tubes for the Preliminary Analysis studies, and the construction of an initial Archaeal 16S rRNA gene clone library were performed by Peggy Law (Figure 2-1).

2.2.2 Cultured MFT from Columns

Samples taken from columns (used in the study of the effects of gas production on increased densification) were used to show the changes in methanogenic communities over time, with and without acetate amendment. The columns were initially equilibrated at 4°C for 85 d, and then transferred to 24°C for 33 d (Guo et al., 2004). Samples taken at an initial time (t=0) 7 d before the columns were warmed up, and a later time (t=end) 7 d after the columns were cooled down to 4°C. These two samples were used to show the changes in the community composition over time in two columns, one amended with acetate and one unamended (Figure 2-1). These samples were used to inoculate methanogen MPN culture tubes for MPN enumeration (performed by Debbi Coy).The MPN culture medium contained acetate as a carbon source for the methanogenic microorganisms, and were incubated for 30 d before they were scored for methane production by gas chromatography. The MPN tubes were stored at 4°C until DNA extraction could be performed.

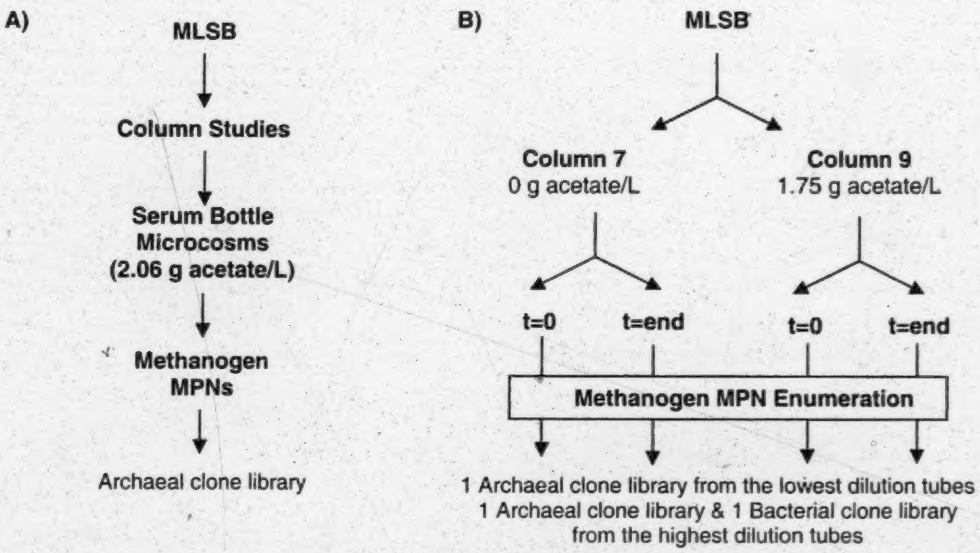

A)
MLSB
Column Studies
Serum Bottle Microcosms (2.06 g acetate/L)
Methanogen MPNs
Archaeal clone library
B)
MLSB
Column 7
0 g acetate/L
Column 9
1.75 g acetate/L
t=0
t=end
t=0
t=end
Methanogen MPN Enumeration
1 Archaeal clone library from the lowest dilution tubes
1 Archaeal clone library & 1 Bacterial clone library from the highest dilution tubes

Figure 2-1: Summary of 16S rRNA gene clone libraries made from the columns. A) Preliminary experiments with tailings incubated in serum bottle microcosms amended with acetate. B) Clone libraries constructed from acetate-amended and unamended columns.

2.2.3 Neat MFT samples from active settling basins

MFT samples were collected from the Central Station in MLSB in May 2004, and Station 1 in WIP in April 2004 (Figure 2-2). The chemical and physical characteristics were determined by Dr. M. MacKinnon of Syncrude (Appendix 7). The samples were stored at 4°C until they were received by our laboratory in June of 2004. Once the samples were received, portions of the MFT were transferred into sterile 125-mL serum bottles leaving no headspace, stoppered, and stored at 4°C. Subsequent samples were removed using an 18-gauge needle and 1-mL syringe to keep the samples anaerobic.

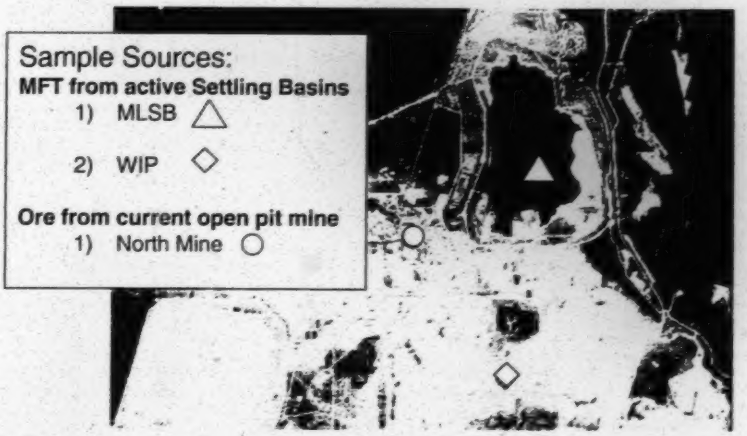

Sample Sources:
MFT from active Settling Basins
1) MLSB △
2) WIP ◇
Ore from current open pit mine
1) North Mine ○

Figure 2-2: Satellite image of Syncrude's Mildred Lake site, indicating locations where tailings and ore samples were obtained.

2.2.4 Oil Sands Ore Samples

Oil sands ore samples were obtained by Brenda Wright from Syncrude's North Mine at the Mildred Lake site on November 23, 2004 (Figure 2-2). Several centimeters of oil sand were removed from the mine face, and a small mass of oil sand was then placed into a sterile jar, and covered with sterile anaerobic 0.1% pyrophosphate buffer containing 0.1% thioglycollate and 10 mL of a suspension of amorphous ferrous sulfide as a reducing agent (Brock and O'Dea, 1977). The reducing agent prevented extended exposure of the oil sand to oxygen, which would decrease the viability of methanogenic species present in the oil sands. Amorphous ferrous sulfide was made by combining equimolar amounts of $Na_2S{\cdot}9H_2O$ (2.7 g) and $Fe(NH_4)_2(SO_4)_2$ (4.41 g) in 125 mL boiling doubly distilled water in a glass-stoppered bottle. The precipitate was allowed to settle, and then washed four times with boiling water to remove all free S^{2-}. The solution was transferred to a 150-mL serum bottle, sparged with N_2 to remove any remaining oxygen, and autoclaved.

Once the oil sand ore samples arrived in the laboratory, they were shaken, and the suspend[illegible]s were transferred into 50-mL serum bottles (leaving no headspace), stoppered and stored at 4°C. Subsequent samples were taken through an 18-gauge needle and 1-mL syringe to keep the samples anaerobic.

2.3 DNA extraction

2.3.1 Preliminary Microcosm Studies

Samples for DNA extraction were taken using a 1 mL syringe and 18 gauge needle from 10^{-1} dilution of the MPN tubes (Section 2.2.1) inoculated from microcosm samples when the headspace had reached 10% (v/v) methane. The MPN culture (300 μL) was suspended in equal volumes of 100 mM phosphate buffer (pH 8), 10% SDS lysis buffer (10% SDS, 0.5M Tris-HCl (pH 8), 0.1M NaCl) and a 24:1 mixture of chloroform:isoamyl alcohol, and 1.0 g zirconia/silica beads (Biospec Products Inc., Bartleville OK, USA; equal amounts of 2.5 mm and 0.1 mm beads). The samples were shaken for 90 s in a Mini-Beadbeater™ (Model 221.BX, Biospec Products, Inc.) at 5000 reciprocations/min. DNA was precipitated from the supernatant using 0.55 volumes of 7 M ammonium acetate. DNA was purified first by an isopropanol precipitation, and then using the GENECLEAN Glassmilk Kit following the manufacturer's protocol (Qbiogene, USA). DNA was eluted in 25 μL sterile water.

2.3.2 Cultured MFT from Columns

The lowest MPN dilution tubes (presumably containing the highest diversity of microorganisms) and the highest methane-positive MPN dilution tubes (presumably containing the most prevalent microorganisms) were used for DNA extraction as described above (Section

2.3.1). DNA was extracted from each of the three tubes from the lowest and highest positive dilutions. The DNA was not pooled at this point, but each of the extracted DNA samples was used separately for PCR amplification (Section 2.4.2).

2.3.3 Optimization of DNA Extraction from Neat MFT

A composite MFT sample consisting of equal volume of MFT from each of four depths of MLSB (6 m, 10 m, 20 m, and 30 m) was used in the optimization experiments. Three methods of DNA extraction were compared to determine which gave best genomic DNA template for 16S rRNA gene amplification. All methods were based on mechanical lysis using a bead-beater. Two kits, Powersoil DNA Isolation kit (Mo Bio Laboratories, Inc, USA) and the FastDNA SPIN kit (for soil) (BIO101 Systems, Germany) were used, as well as our own protocol of mechanical lysis in the presence of SDS-chloroform-isoamyl alcohol (Section 2.3.1). As well, incubation at 70°C for 10 min before mechanical lysis for each of the extraction protocols was attempted to see if this would result in more efficient cell lysis. All extractions were performed first using the Mini-Beadbeater™ at 5000 reciprocations/min, and then with the Fast Prep Cell Disrupter (BIO101 Systems, Germany).

The presence of amplifiable DNA template was evaluated in all cases by amplification of Archaeal and Bacterial 16S rRNA genes by PCR, as described in Section 2.4.2. Twenty-five microliters of each PCR reaction were separated on a 1% agarose gel, stained with ethidium bromide and visualized by UV illumination. The intensities of the bands were compared by eye to determine which method of DNA extraction allowed for the highest level of PCR amplification.

It was determined that the FastDNA SPIN kit (for soil) provided the highest quality of genomic DNA template with the least occurrence of contamination (Section 3.3.3), so this was the protocol that was used for further DNA extraction from the tailings. Using this kit, different speeds of beadbeating using the Fast Prep (BIO101 Systems) beadbeater were compared to allow for maximum yield with minimum shearing. This work was performed by Marshneil Chandra. The best speed was determined to be 6.0 m/s for 30 s (Section 3.3.3), and this was the setting used for all subsequent DNA extractions.

2.3.4 DNA Extraction from Neat MFT

Genomic DNA was extracted from the tailings using the FastDNA SPIN Kit (for soil) following the manufacturer's protocol except that cells were disrupted at 6.0 m/s for 30 s. Duplicate replicate extractions were performed for each tailings sample. The DNA was eluted in 50 μL of sterile water and stored at -20°C. The samples were not pooled at this point, but were each used separately for PCR amplification (Section 2.4.3).

2.3.5 Optimization of DNA extraction from the Oil Sands Ore

Small scale extractions were performed using the FastSPIN DNA extraction kit (for soil) following the manufacturer's protocol with the modifications described in Section 2.3.4. Oil sands ore sample #3 was used for these experiments due to its high fines content (Table 3-11). Two different samples from the oil sands ore suspension were used for DNA extraction: 0.3 mL of the fine tails suspension, and ~0.5 g of the coarse sand taken from the bottom of the jar. MPN culture from Column 7 (t=end) 10^{-1} dilution was used as a positive control, and a mixture of 0.1 mL MPN culture and 0.2 mL fine tails suspension from the oil sand in buffer was used to determine if residual hydrocarbons could be removed to produce a clean DNA template.

Large scale extractions were performed using the PowerMax™ Soil DNA Isolation Kit (Mo Bio Laboratories) following the manufacturer's protocol. Oil sands ore sample #4 was used for these experiments. Two samples from the oil sands were used for DNA extraction: 10 mL of the fine tails suspension, and 10 g of the unsuspended oil sand provided by M. MacKinnon in the fall of 2003. MPN culture from Column 7 (t=end) 10^{-1} dilution was used as a positive control, and a mixture of 0.5 mL MPN culture and 10 mL fine tails suspension from the oil sand in buffer was used to determine if contaminating hydrocarbons could be removed to produce a clean DNA template.

2.4 Amplification of 16S rRNA genes

2.4.1 Preliminary Microcosm Studies

Archaeal 16S rRNA genes were amplified from the extracted DNA using primers 21F (5'-TTC CGG TTC ATC CYG CCG A-3') and 958R (5'-YCC GGC CTT GAM TCC AAT T-3') (DeLong, 1992). All molecular reagents and enzymes were purchased from Roche Diagnostics GmbH, Manheim, Germany unless otherwise stated. All primers were synthesized by the Molecular Biology Services Unit in the Department of Biological Sciences, University of Alberta. PCR reactions (25 μL) contained 1X PCR buffer (10 mM Tris-HCl, 250 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 200 μM each dNTP, 0.2 μM of each primer (21F and 958R), 2.5 U *Taq* DNA polymerase, and 1 μL extracted genomic DNA. The PCR reaction steps consisted of an initial denaturation step at 94°C for 5 min, followed by 27 cycles of 94°C for 30 sec, 51°C for 30 sec, and 72°C for 2 min, followed by a final extension step at 72°C for 8 min. PCR amplification products were separated by gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and visualized by UV illumination. The expected size of the amplified Archaeal 16S rRNA gene fragments was approximately 950 bp.

2.4.2 Cultured MFT from Columns

Archaeal 16S rRNA genes were amplified from the extracted genomic DNA using the primers 21F and 958R (DeLong, 1992). Each 25 μM PCR reaction contained 1X PCR buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mM BSA, 10 mM $(NH_4)_2SO_4$, 200 μM each dNTP), 1 μM of each of the primers 21F and 958R, 2.5 U *Taq* DNA polymerase, and 5 μL of purified genomic DNA. The same PCR conditions were used as described in Section 2.4.1. Three replicate PCR reactions were performed on each of the three DNA extractions to eliminate PCR bias and to increase the total yield of amplified PCR product. The amplified fragments (expected size of ~950 bp) were separated by electrophoresis on a 1% agarose gel, stained with ethidium bromide, and visualized by UV illumination.

Bacterial 16S rRNA genes were amplified from the extracted genomic DNA using the primers PB36 (5'-AGR GTT TGA TCM TGG CTC AG-3') and PB38 (5'-GKT ACC TTG TTA CGA CTT-3') (Saul et al., 2005). Each 25 μL PCR reaction contained 1X PCR buffer (same as for Archaeal PCR), 0.4 μM of each of the primers PB36 and PB 38, and 1 U of *Taq* DNA polymerase. The PCR program consisted of an initial denaturation at 94°C for 4 min, followed by 30 cycles of 93°C for 45 s, 54°C for 1 min, and 73°C for 2 min, followed by a final extension at 73°C for 10 min. The amplified 16S rRNA gene fragments were separated by electrophoresis on a 1% agarose gel, (expected size of ~1500 bp), stained with ethidium bromide and visualized by UV illumination.

The replicate amplifications of 16S rRNA genes from each sample were pooled at this point, and subsequently cloned into plasmid vectors for construction of a clone library (Section 2.5.2).

2.4.3 Neat MFT Samples from Active Settling Basins

Archaeal and Bacterial 16S rRNA gene fragments were amplified from the extracted DNA as described above for the MPN cultured tubes (Section 2.4.2). Three replicate PCRs were performed on each replicate extracted DNA sample and subsequently pooled to ensure an equal representation of 16S rRNA gene sequences in the sample.

2.4.4 Oil Sands Ore Samples

Quality and quantity of the DNA template was evaluated in all cases by amplification of Bacterial 16S rRNA genes, as described in Section 2.4.2. The PCR-amplified products were separated on a 1% agarose gel, and stained with ethidium bromide and visualized by UV illumination. Band densities were used as a visual measure of DNA yield and quality.

2.5 Cloning and ARDRA

2.5.1 Preliminary Microcosm Studies

PCR products were purified from the agarose gel fragments with the High Pure PCR Purification Kit (Roche Diagnostics GmbH), and cloned into the pGEM-T Easy Vector System

(Promega, Madison WI, USA) following the supplied protocol. Clones were screened by blue/white screening, and 94 white colonies and two blue colonies were picked to create a 16S rRNA gene clone library. Clones were picked using sterile toothpicks and inoculated into 200 mL LB+100 mg ampicillin/L in a 96-well round-bottom culture plate (Corning, Inc, NY, USA).

The 16S rRNA gene fragment was reamplified from each clone using the vector-specific primers M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CAG CAA ACA GCT ATC AC-3'). Plated cells from each clone were resuspended in 20 μL sterile water, and 5 μL of this cell suspension was used as template DNA in the PCR reaction. Each 50-μL reaction contained 1X PCR buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.4 mM β-mercaptoethanol, 0.1 mM BSA, 10 mM $(NH_4)_2SO_4$, 200 μM each dNTP) 1 mM each M13F and M13R primers, and 2.5 U *Taq* DNA polymerase. A modified version of the PCR amplification by Huber et al. (2002) was used. An initial denaturation at 94°C for 5 min was followed by 2 cycles of 94°C for 90 s, 56°C for 45 s, and 72°C for 90 s. This was followed by 22 cycles of 90°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by a final extension step of 72°C for 10 min. A 3-μL portion of each PCR product was visualized on a 1% agarose gel, with an expected fragment size of 1200 bp. Each clone was screened by digestion with *Hae*III and *Cfo*I. The digestion reactions consisted of 1X reaction buffer, 10 U of the restriction enzyme, 0.16 mg BSA/mL, and 17 μL of the PCR product, and were incubated at 37°C for 2 to 4 h. The resulting fragments from each restriction digestion reaction were separated by electrophoresis through a 2% agarose gel in Tris-acetate EDTA buffer at 2.75 V/cm for 3 hours, and then stained with ethidium bromide and visualized by UV illumination.

The patterns of bands were grouped by eye, and one representative clone from each pattern type was selected for sequencing using the M13F and M13R primers. A 50-μL PCR reaction was set up using M13F and M13R primers and resuspended colonies as described above. The amplified DNA product was purified using the High Pure PCR Purification Kit, and eluted in a final volume of 25 μL. Each 20 μL sequencing reaction contained 20-μL BigDye Terminator v3.1 premix (1/4 strength) (PE Applied Biosystems, Foster City CA), 80 mM Tris-HCl, 2 mM $MgCl_2$, 4 pmol of either M13F or M13R primer, and 1 μL of purified DNA template (50 to

100 ng). The PCR program consisted of 25 cycles of 96°C for 30 s, 51°C for 15 s, and 60°C for 60 s. Sequence reactions were cleaned up by adding 0.3 M sodium acetate and 25 mM EDTA to the 20-µL sequencing reaction, then precipitating with ethanol. Sequencing was done on an Applied Biosystems 373A automated DNA sequencer (Applied Biosystems Inc., USA), and the chromatograms from the sequencing reactions were analyzed using the Staden software Package (v. 2003.0 beta) (Dear and Staden, 1991). Sequences were compared to GenBank (http://www.ncbi.nlm.nih.gov/BLAST) for identification of closely related species. The Ribosomal Database Project II (RDP II) was not used for identification of related species due to the low representation of Archaeal sequences in this database. Chimeric sequences were detected with RDP Chimera_Check and were excluded from further analysis.

2.5.2 Cultured MFT from Columns

PCR products were purified from the agarose gel using the QIAquick Gel Purification Kit (Qiagen Inc., Mississauga, ON) and cloned into the pCR®2.1-TOPO vector using the pCR®2.1-TOPO cloning kit (Invitrogen, Carlsbad CA) following the supplied protocol, with the following modifications: (a) the maximum volume (4 µL) of insert DNA was added, and (b) the ligation reaction was incubated at room temperature for 20 to 30 min (instead of 5 min) to ensure maximum cloning efficiency. Ligated plasmids were transformed by heat-shock into OneShot® TOP10 chemically competent *E. coli* cells and plated onto Luria-Bertani (LB) agar plates (Per L: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15g agar, pH 7.5) containing 50 mg kanamycin/L. Clones were screened by blue/white screening, and white colonies were picked to create a 16S rRNA clone library. Archaeal 16S rRNA clone libraries consisted of 85-95 clones, and Bacterial clone libraries consisted of 85 to 190 clones. The size of the clone libraries used was based on initial observations of pattern diversity, and more clones were picked if deemed necessary to improve clone coverage of the sample diversity. Clones were picked with sterile toothpicks and were inoculated into 100 µL LB + 50 mg kanamycin/L in a 96-well culture plate (Corning, Inc.). The culture plates were incubated at 37°C overnight with shaking at 150 rpm. For short-term storage of the clone libraries, 2.5 µL of each culture was spotted onto LB agar+50 mg kanamycin/L and grown overnight at 37°C, then stored at 4°C. For long term storage of the clone libraries, 50 μL of 45% glycerol was added to each well of the culture plate for a final concentration of 15% glycerol, and the plates were sealed in plastic bags and stored at -80°C.

The 16S rRNA gene fragment was reamplified from each clone as described in Section 2.5.1. A 2 μL portion of each PCR product was visualized on a 1% agarose gel, with an expected fragment size of ~1200 bp for the Archaeal libraries, and ~1700 bp for the Bacterial libraries. Each clone was screened by digestion with *Hae*III and *Cfo*I as described in Section 2.5.1 except the amount of enzyme used was reduced to 5 U.

Fragment sizes were determined using GelPro Analyzer Software version 4.5 (Media Cybernetics, Inc) compared to Molecular Weight Marker XIV (Roche Diagnostics GmbH). Patterns were grouped based on both visual inspection and comparison of fragment sizes. One clone from each representative pattern was sequenced using M13F and M13R primers for Archaeal clones; and M13F, M13R and 5R (5'-GCT CGT TGC GGG ACT TAA C-3')(Foght et al., 2004) primers for Bacterial clones, as described in Section 2.5.1. Sequences were aligned and compared to databases as in Section 2.5.1 except that the Ribosomal Database Project II (RDP II) (Maidak et al., 1994) was used in addition to GenBank for comparison of Bacterial sequences.

2.5.3 Neat MFT Samples from Active Settling Basins

Bacterial and Archaeal 16S rRNA clone libraries were constructed for each of the tailings samples as described previously (Section 2.5.2). Archaeal clone libraries consisted of 90 to 94 clones plus one blue colony (negative control), and Bacterial clone libraries consisted of 90 to 188 clones plus two blue colonies (negative controls). The size of the clone libraries used was based on initial observations of pattern diversity, and more clones were picked if deemed necessary to improve clone coverage of the sample diversity.

2.6 Phylogenetic Analyses

Cloned 16S rRNA gene sequences were aligned with their nearest neighbors (obtained from GenBank) using ClustalX, and phylogenetic analysis was done using Phylip Version 3.63.

Trees were constructed using a Kimura 2-parameter distance matrix based Neighbor-Joining method, using either *E. coli* (for Archaeal trees) or *Methanosarcina barkeri* (for Bacterial trees) as an outgroup and bootstrapped using 100 replications.

2.7 Methanogen Enumeration

2.7.1 Neat MFT Samples from Active Settling Basins

Methanogenic species in the tailings were enumerated using the three-tube MPN enumeration method. Bicarbonate methanogenic medium (Holowenko et al., 2000) contained (per L) 4.0 g sodium hydroxide, 2.0 g Difco yeast extract (BDL), 2.0 g trypticase peptones, 0.5 g 2-mercaptoethanesulfonic acid (sodium salt), 1.4 mg resazurin, 14 mL of Mineral Solution I (per 100 mL: 5.0 g NaCl, 1.0 g $CaCl_2\cdot 2\ H_2O$, 1.0 g NH_4Cl, 1.0 g $MgCl_2\cdot 6H_2O$, and 0.01 mM HCl) and 1.4 mL Mineral Solution II (Per 100 mL: 1.0 g $(NH_4)_{6\,m}o_7O_{24}\cdot 2H_2O$, 0.01 g $ZnSO_4\cdot 7H_2O$, 0.03 g H_3BO_3, 0.15 g $FeCl_2\cdot 4H_2O$, 1.0 g $CoCl_2\cdot 6H_2O$, 0.003 g $MnCl_2\cdot 4H_2O$, 0.003 g $NiCl_2\cdot 6H_2O$, 0.01g $AlK(SO_4)\cdot 12H_2O$), and 6.805 g sodium acetate. The pH was adjusted to pH 7.2 to 7.4 by sparging with O_2-free 30% CO_2 balance N_2. Each MPN culture tube contained 9 mL acetate-bicarbonate medium, and 0.1 mL vitamin B solution (Per L: 0.1 g nicotinic acid, 0.1 g cyanocobalamine (B12), 0.05 g thiamine, 0.05 g p-aminobenzoic acid, 0.25 g pyridoxine, 0.025 g/L panthothenic acid; (Fedorak and Hrudey, 1984)) and 0.25% sodium sulfide monohydrate were added just prior to inoculation. Tailings were 10-fold serially diluted to 10^{-8} and 1 mL of each dilution was inoculated into each of three MPN tubes.

The MPN tubes were incubated for 30 d at room temperature in the dark. Methane in the headspace was detected a Hewlett Packard 5700A gas chromatograph and a flame ionization detector with a 2mx0.3cm column packed with TenaxGC (60/80 mesh). N_2 was used as the carrier gas at 50 mL/min, H_2 was set at 50 mL/min and air at 200 nlL/min. The temperatures of the injector, oven and detector were set at 30°C, 30°C and 200°C, respectively. Chromatographs and peak areas were determined using a Hewlett Packard 3380A integrator (Holowenko et al., 2000). Tubes containing over 0.1% (v/v) methane were considered positive.

2.7.2 Oil Sands Ore

Methanogens were enumerated as described above for the tailings (Section 2.7.1).

2.8 SRB Enumeration

2.8.1 Neat MFT Samples from Active Settling Basins

Sulfate-reducing bacteria in the tailings were enumerated using the three-tube MPN enumeration method. A modified Butlin's medium was used, containing (per L) 0.5 g K_2HPO_4, 1.0 g NH_4Cl, 2.0 g Na_2SO_4, 0.067 g $CaCl_2 \cdot 2H_2O$, 1.0 g $MgSO_4 \cdot 7\ H_2O$, 1.0 g Difco Yeast Extract, trace $FeSO_4 \cdot 7H_2O$, 1 mg/L resazurin, and 0.15% sodium lactate, and the pH was adjusted to 7.5. Each MPN culture tube contained 9 mL Butlin's medium and two iron nails, precleaned with HCl. Tubes were inoculated with 1 mL tailings that had been serially 10-fold diluted to 10^{-8}.

The MPN tubes were incubated for 30 d at room temperature in the dark. Positive growth of SRB was indicated by a black precipitate formed on the iron nails at the bottom of the tube.

2.8.2 Oil Sands Ore

SRB species were enumerated by MPN as described above for the neat MFT (Section 2.8.1).

2.9 Methane Production from Neat MFT

Fifty milliliters of tailings were transferred aseptically into 125-mL serum bottles. The bottles were sealed and flushed with 30% CO_2 balance N_2. The microcosms were incubated at room temperature in the dark, and headspace methane concentrations were measured approximately every 14 days for approximately 200 days by gas chromatography (Section 2.7.1).

3 Results and Discussion

The results are presented in four major sections. First the results from the preliminary microcosm studies are discussed followed by the examination of cultured MFT from acetate-amended and unamended columns from laboratory studies (Sections 3.1 and 3.2 respectively). The second set of results involve the analysis of neat MFT from settling basins and the analysis of the oil sands ore (Sections 3.3 and 3.4, respectively), both performed without prior cultivation.

3.1 Preliminary Studies

MFT samples were taken from laboratory-incubated columns in 2003 and incubated anaerobically in serum bottles with acetate amendment. MPN tubes inoculated with this anaerobic culture were used for DNA extraction and construction of an Archaeal 16S rRNA clone library. This sample was used to develop the methods used for the rest of the experiments, and to estimate the level of diversity of methanogens present in the tailings.

Ninety-one clones were screened by restriction digestion, and the frequency of each OTU observed is shown in Figure 3-1. (Each OTU corresponds to a unique ARDRA pattern; Appendices 1 to 3). Three dominant OTUs (A12, A15 and A16, representing a total of 50% of clones) were observed, as well as nine less dominant OTUs consisting of two or more representatives (representing 34% of the clone library). Fourteen OTUs were represented only once in the clone library, indicating that these may be sequences from microorganisms which were not very dominant in the acetate-amended cultures.

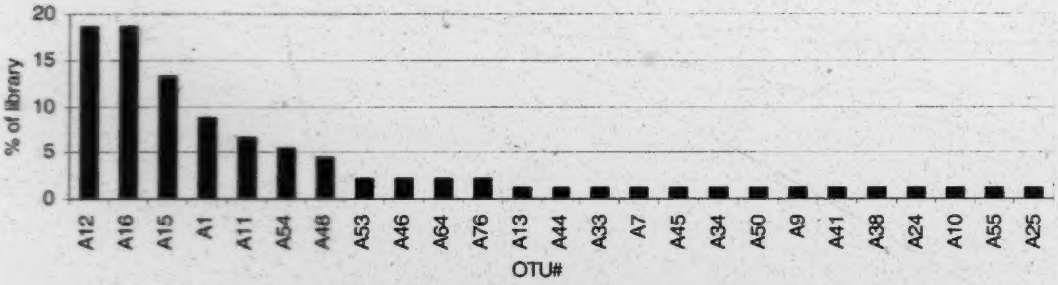


Figure 3-1: Frequency of ARDRA patterns observed in an Archaeal 16S rRNA gene clone library constructed from the lowest dilution MPN cultures inoculated with acetate-amended microcosm tailings culture.

At least one representative clone from every OTU was selected for sequencing, with two clones sequenced from the most dominant patterns. These sequences were compared to the GenBank database for identification of the most closely related species (Appendix 3). The number of OTUs, the % of the library related to each family of methanogens and the most closely related species to each OTU is shown in Table 3-1. Sequences were not compared to the RDP II database because Archaeal 16S rRNA gene sequences are not well represented in that database. Three dominant OTUs (A1, A11 and A12) and three non-dominant patterns (totaling 37.4% of the library) represented cloned sequences related to *Methanocorpusculum* spp. Two dominant OTUs (A15 and A16) and six non-dominant OTUs (totaling 38.5% of the library) represented cloned 16S rRNA gene sequences related to the genus *Methanocalculus*. Both *Methanoculleus* and *Methanocorpusculum* are reported to use H_2+CO_2 and formate as substrates for methane production, but are unable to use acetate. In addition, there were five clones related to *Methanoculleus* species, which are also hydrogenotrophic methanogens unable to use acetate.

In total, over 80% of the clones in this clone library had sequences related to hydrogenotrophic methanogens, using H_2 to reduce either CO_2 or formate to methane, despite the fact that the library was constructed from a methanogenic microcosm amended with acetate. Clones related to *Methanosarcina* and *Methanosaeta* species were represented by 16.5% and 2.2% of the clone library, respectively. *Methanosarcina* spp. are able to utilize H_2+CO_2, as well as acetate and methanol, whereas *Methanosaeta* spp. are obligate acetoclastic methanogens. Clones related to *Methanomethylovorans* spp. made up 2.2% of the library. These microorganisms grow methanogenically on methanol and methylamines, but are unable to use any of the more common substrates H_2+CO_2, formate, or acetate.

The dominance of sequences related to hydrogenotrophic methanogenic species indicates that the added acetate is not being directly consumed by most methanogens in the MPN tubes, but it may be first cleaved by bacterial species to produce H_2+CO_2 or formate which can then be used by the methanogens. The analysis of bacterial species in these cultures might have indicated what bacterial species are important for producing substrates for the methanogens, but the Bacterial community was not examined in this preliminary study.

Table 3-1: Species most closely related to partial sequences of Archaeal 16S rRNA gene clones. This clone library was constructed using DNA extracted from MFT amended with acetate and incubated in an anaerobic serum bottle microcosm.

Genus	OTU	# clones (% of library)	Closest Related Species	% Sequence Similarity (GenBank)	Reported Carbon Source Used[a] (indicated for each genus)						
					H_2+CO_2	for	2-prop	2-but	ac	MeOH	$MeNH_2$
Methanocorpusculum	A1	8	*M. labreanum* strain DSM 4855; AY260436	97	•	•					
	A7	1	*M. parvum* strain DSM 3829; AY260435	95							
	A9	1	*M. parvum* strain DSM 3829; AY260435	97							
	A10	1	*M. parvum* strain DSM 3829; AY260435	97							
	A11	6	*M. parvum* strain DSM 3829; AY260435	96							
	A12	17	*M. parvum* strain DSM 3829; AY260435	96							
Total		**34 (37.4%)**									
Methanocalculus	A13	1	*M. pumilus*; AB008853	97	•	•	•				
	A15	12	*M. pumilus*; AB008853	98							
	A16	17	*M. pumilus*; AB008853	97							
	A24	1	*M. pumilus*; AB008853	97							
	A25	1	*M. pumilus*; AB008853	97							
	A33	1	*M. taiwanese* strain P2F9704a; AF172443	97							
	A34	1	*M. taiwanese* strain P2F9704a; AF172443	98							
	A38	1	*M. taiwanese* strain P2F9704a; AF172443	98							
Total		**35 (38.5%)**									
Methanoculleus	A41	1	*M. chikugoensis*; AB03795	96	•	•	•	•			
	A44	1	*M. palmeoli*; Y16382	97							
	A45	1	*M. palmeoli*; Y16382	97							
	A46	2	*M. palmeoli*; Y16382	96							
Total		**5 (5.5%)**									
Methanosarcina	A48	4	*M. acetivorans*; M59137	98	•				•	•	•
	A50	1	*M. mazei* strain Goe1; AE013440	97							
	A53	2	*M. mazei* strain MT; AY260432	98							
	A54	5	*M. mazei* strain MT; AY260432	96							
	A55	1	*M. mazei* strain MT; AY260432	98							
Total		**13 (14.3%)**									
Methanomethylovorans	A58	2	*M. hollandica* strain ZB (AF120163)	99						•	•
Total		**2 (2.2%)**									
Methanosaeta	A70	2	*Methanosaeta* sp. Clone A1 (AJ133791)	99					•		
Total		**2 (2.2%)**									

[a] Abbreviations used: for, formate; 2-prop, 2-propanol + CO_2; 2-but, 2-butanol + CO_2; ac, acetate; MeOH, methanol; $MeNH_2$, methylamines

3.1.1 Coverage of the Archaeal clone library

An accumulation curve plotting the number of OTUs *versus* the number of clones sampled from the Archaeal clone library (Figure 3-2) approaches a horizontal line, indicating that the library contains sufficient clones to represent the true diversity present in the sample. The curve is asymptotic, indicating that sampling more clones would not likely result in the discovery of many more new patterns (Kemp and Aller, 2004). The coverage of this library was calculated to be 0.85 using Good's Coverage calculation (Equation 1-6) (Good, 1953).

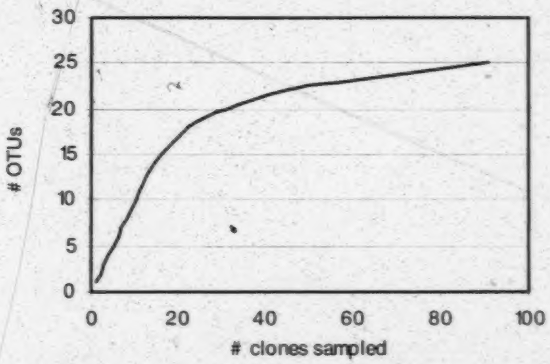


Figure 3-2: Accumulation curve for the Archaeal clone library constructed from the acetate-amended tailings culture.

The interpretation of clone library coverage is fairly subjective; there is no "acceptable" value, however the published coverage values for aquatic sediments tend to fall in the range of 0.70 to 0.95 (Kemp and Aller, 2004). Therefore, the coverage for this Archaeal library is in an acceptable range for aquatic environmental samples, indicating that the clone library adequately describes the Archaeal diversity present in the sample analyzed. This cultured sample is probably much less complex than a sample directly taken from the environment, and therefore it is easy to obtain clones representing all of the species present, and indicates which species are important in methane production from acetate in the cultured tailings.

The species richness, as calculated by the Shannon-Weaver index, was 2.62, indicating that the sample was fairly diverse. An exhaustive search of the literature has revealed that there are no set standards for describing diversity in an environment because it depends on the criteria used to define an OTU (i.e.: based on sequence similarity or based on similar ARDRA pattern). Diversity indices are most useful in comparing the diversity between samples in the same study, when the same definition of an OTU is used.

3.2 Effects of Acetate Amendment on Archaeal and Bacterial Populations

3.2.1 Archaeal Clone Library Results

Because an acetate-amended methanogenic consortium was used to inoculate the MPN tubes used for clone library construction in the preliminary studies, it is not knows how the community may differ from the original community before acetate-amendment. To examine this further, MFT samples were taken from column studies (used to study the effects of acetate amendment on gas production) at the initial and final time points from either acetate-amended or unamended columns, and used to inoculate methanogenic MPN culture tubes.

16S rRNA clone libraries were constructed from DNA extracted from either the lowest dilution or the highest methane-positive dilution tubes. The data from the highest methane-positive MPN tubes presumably will represent the most dominant methanogenic species present in the original sample because less dominant species will have been diluted to extinction. On the other hand, the data from the lowest dilution tubes will better indicate the diversity of methanogens present in the original sample. However, only tentative conclusions can be made regarding the relative proportions of the microbes found in the MPN tubes because the populations also will have changed to favor those species which can grow best in the methanogenic medium provided.

Figure 3-3 shows the frequency of each OTU in the 16S rRNA clone libraries constructed from the highest positive dilution tubes, presumably representing the most dominant methanogenic species in the columns. In Column 7 (unamended) approximately 85% of the clones were represented by only three OTUs. The distribution of these patterns did not change between the two time points, which is not surprising because the tailings were not amended and the conditions should not have selected for or against any methanogenic species.

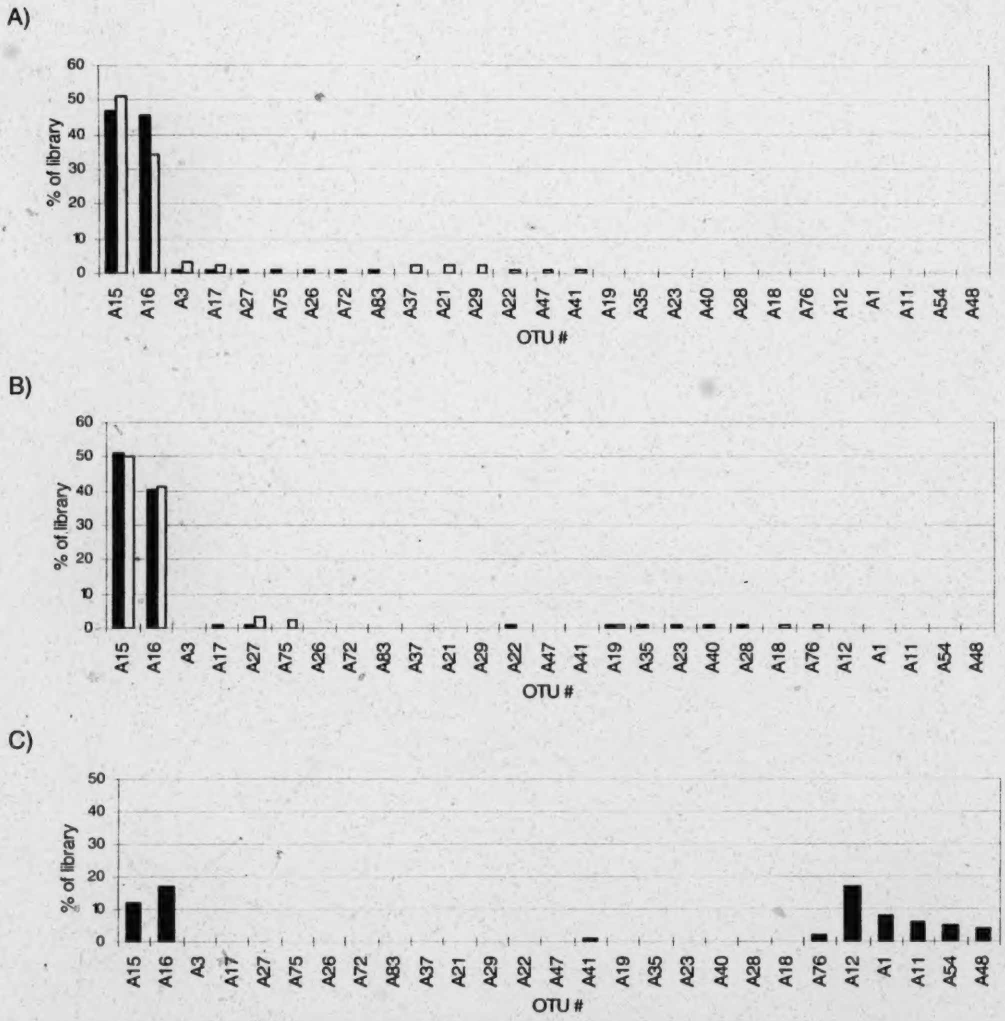


Figure 3-3: ARDRA patterns obtained from Archaeal 16S rRNA gene clone libraries constructed from the highest dilution MPN culture tubes positive for methane production, and presumably representing the dominant methanogens in the columns at t=0 (black bars) and t=end (grey bars). A) Column 7 (0 g acetate/L), B) Column 9 (1.75 g acetate/L), C) Some OTUs found in the preliminary studies of a clone libraries constructed from tailings microcosm amended with 2.06 g acetate/L (Section 3.1).

However, the same general trend also was observed for Column 9, which was amended with 1.75 g acetate/L MFT. Four OTUs represented approximately 90% of the clones in the library, and the distribution did not dramatically change over time. This is surprising because the chemical results from the column studies showed that the addition of acetate to the tailings enhanced methane production (R. Chalaturnyk, personal communication). Because more methane was evolved from the amended columns, it is expected that the acetate amendment would have caused changes in the methanogenic community over time by selecting for those that are readily able to use acetate as a carbon source.

Figure 3-3 (C) shows the OTUs observed in the libraries constructed from the high dilution tubes inoculated with MFT from the acetate-amended and unamended columns, and compares them to some of the OTUs observed in the preliminary studies involving acetate amended tailings microcosms. The same two dominant OTUs are observed (A15 and A16) in all libraries, however the clone library constructed from the acetate-amended microcosm (Section 3.1) also contains five dominant patterns which were not observed in the libraries constructed from the columns. This may mean that there were different methanogenic species present in the samples used in the construction of the initial Archaeal 16S rRNA clone library, that were not present in the MFT used in the column studies.

Figure 3-3 (A) and (B) presumably represent the dominant OTUs present in the column MFT, whereas Figure 3-4, constructed from the lowest dilution MPN tubes, presumably better represents the true diversity of methanogens in the MFT samples. This is reflected in the higher number of OTUs seen in Figure 3-4. The distribution of less dominant OTUs changes in the libraries constructed from the lowest dilution tubes (Figure 3-4), which is not observed in the libraries constructed from the highest dilution tubes (Figure 3-3). Several patterns appear only in the t=end sample from Column 7 (A1, A11, A50, A52 and A80) and some appear only in the t=end sample from Column 9 (A22, A26, A29, A34, A48, A51, A78, A79). These OTUs may represent species of methanogens that are either stimulated by acetate, or grow better without acetate-amendment.

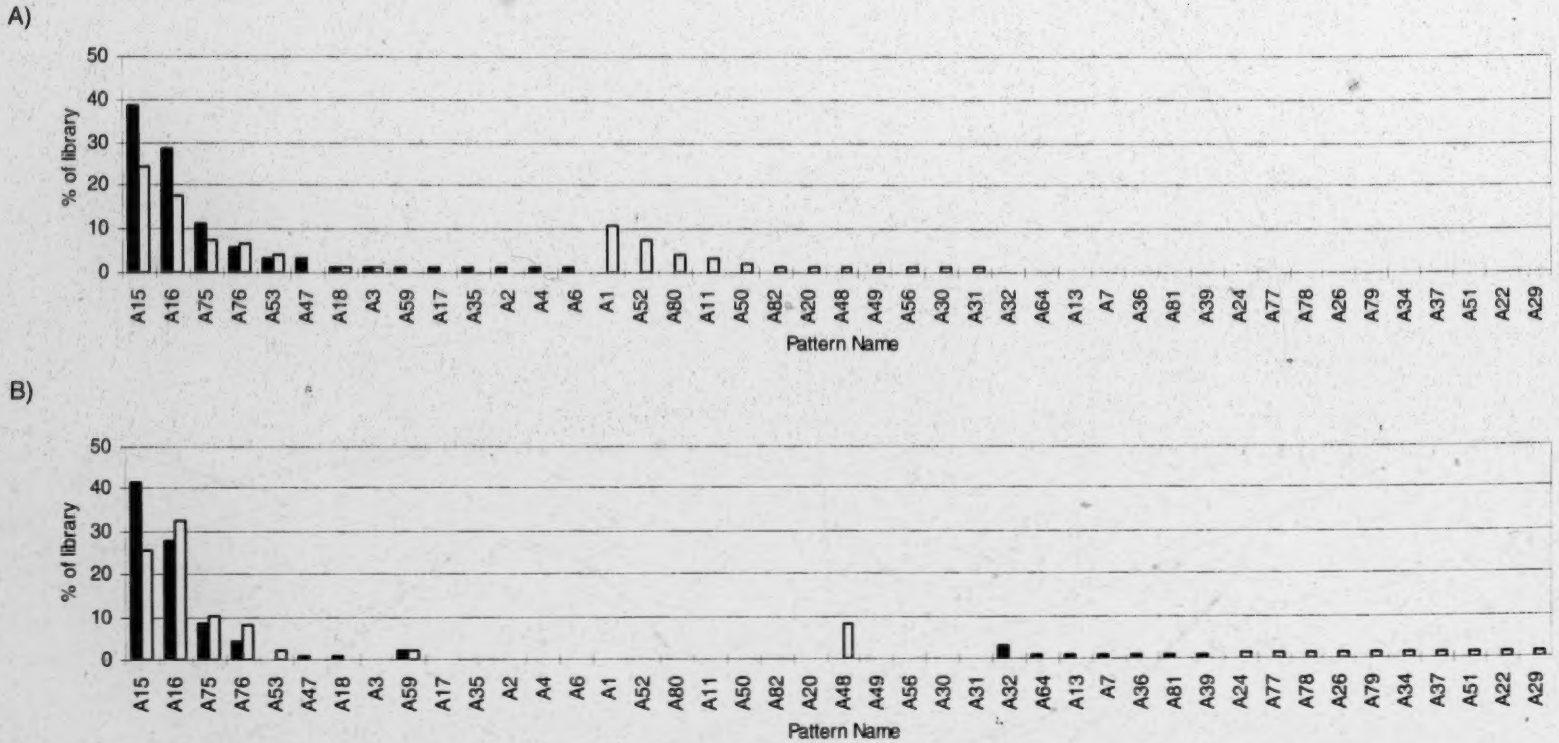


Figure 3-4: Diversity of ARDRA patterns obtained from Archaeal 16S rRNA gene clone libraries constructed from the lowest dilution MPN culture tubes and presumably representing total diversity of methanogens in the columns at t=0 (black bars) and t=end (grey bars). A) Column 7 (0 g acetate/L), B) Column 9 (1.75 g acetate/L).

After grouping clones into OTUs based on their ARDRA patterns, one clone from each representative OTU was sequenced and the sequence compared to those in GenBank. The frequencies of each OTU in each of the clone libraries are shown in Table 3-2 (high dilution MPN tubes) and Table 3-3 (low dilution MPN tubes). The methanogenic clones representing each OTU appeared to have sequences which were very similar (95 to 99%) to known methanogen sequences (Appendix 3) in the GenBank database, and therefore could be further grouped based on genus (Table 3-4). The clusters of similar sequences are obvious in the phylogenetic tree (Figure 3-5). A close relationship to previously isolated species indicates that these methanogenic species represented by the clones in the study are not unusual or specific to the tailings environment, but may be commonly found in other anaerobic environments.

Similar species of methanogens were found in the acetate-amended and unamended MFT from the columns to those found in the acetate-amended microcosms from the preliminary studies (Section 3.1). This may simply indicate that certain species are better able to grow in the conditions provided in the laboratory (room temperature, acetate amendment), than others. A greater diversity of OTUs was found in these studies than in the preliminary studies, suggesting that there is a greater diversity of methanogens in the column tailings than in the serum bottle microcosms, perhaps because the serum bottle microcosms have undergone an additional culture step compared to the MFT from the columns.

Table 3-2: Number of clones with sequences related to methanogenic Archaea in clone libraries constructed from the highest dilution MPN culture tubes inoculated with MFT taken from acetate-amended or unamended columns.

		Number of Clones			
		Column 7 (no acetate)		**Column 9 (acetate)**	
	OTU	**T=0**	**T=end**	**T=0**	**T=end**
Methanomicrobiales					
Methanocorpusculum	A3	1	3		
Total		**1**	**3**	**0**	**0**
Methanocalculus	A15	43	48	48	45
	A16	42	32	38	37
	A17	1	2	1	
	A18				1
	A19			1	1
	A21		2		
	A22		1	1	
	A23			1	
	A26	1			
	A27	1		1	3
	A28			1	
	A29		2		
	A35			1	
	A37		2		
	A40			1	
Total		**88**	**89**	**98**	**87**
Methanoculleus	A41		1		
	A47		1		
Total		**0**	**2**	**0**	**0**
Methanosarcinales					
Methanosaeta	A72	1			
Total		**1**	**0**	**0**	**0**
Methanomethylovorans	A75	1			2
	A76				1
Total		**1**	**0**	**0**	**3**
Methanobacteriales					
Methanobrevibacter	A83	1			
Total		**1**	**0**	**0**	**0**
Clone library size		**94**	**94**	**94**	**90**

Table 3-3: Number of clones with sequences related to methanogenic Archaea in clone libraries constructed from the lowest dilution MPN culture tubes inoculated with MFT taken from acetate-amended or unamended columns.

		Number of Clones			
		No Acetate		Acetate	
	OTU	T=0	T=end	T=0	T=end
Euryarchaeota					
Methanomicrobiales	A1		10		
Methanocorpusculum	A2	1			
	A3	1	1		
	A4	1			
	A5				1
	A6	1			
	A7			1	
	A11		3		
Total		**4**	**14**	**1**	**1**
Methanocalculus	A13			1	
	A15	34	22	36	22
	A16	25	16	29	28
	A17	1			
	A18	1	1	1	
	A20		1		
	A22				1
	A24			1	1
	A26			1	
	A29				1
	A30		1		
	A31		1		
	A32		1		
	A34				1
	A35	1			
	A36			1	
	A37				1
	A39			1	
Total		**62**	**43**	**71**	**55**
Methanoculleus	A47	3		1	
Total		**3**	**0**	**1**	**0**
Methanosarcinales	A48		1		7
Methanosarcina	A49		1		
	A50		2		
	A51				1
	A52		7		
	A53	3	4		2
	A56		1		
Total		**3**	**16**	**0**	**10**
Methanosaeta	A59	1		2	2
	A64			3	
Total		**1**	**0**	**5**	**2**
Methanomethylovorans	A75	10	7	8	9
	A76	5	6	4	7
	A77			1	
	A78			1	
	A79			1	
	A80		4		
Total		**15**	**17**	**15**	**16**
Methanolobus	A81			1	2
Total		**0**	**0**	**1**	**2**
Methanobacteriales					
Methanobacterium	A82		1		
Total		**0**	**1**	**0**	**0**
Clone Library Size		**88**	**91**	**94**	**86**

Table 3-4: Species most closely related to nearly full length sequences of Archaeal 16S rRNA clones. The clones were obtained from clone libraries constructed from methanogenic cultures inoculated with MFT taken from acetate-amended or unamended columns.

Putative Genus	#OTUs	Closest Related Species	% Sequence Similarity (GenBank)	Reported Carbon Source Used[a] (indicated for each genus)						
				H_2+CO_2	for	2-prop	2-but	ac	MeOH	$MeNH_2$
Methanomicrobiales										
Methanocorpusculum	12	*M. parvum* DSM 3828 (AY260435)	95-98	•	•	•				
		M. labreanum strain DSM 4855 (AY260436)	95-97							
		Symbiont of *Trimyema compressa*	98-99							
Methanocalculus	31	*M. halotolerans* strain P2F9705 (AF411470)	95-97	•	•					
		M. pumilis (AB008853)	96-98							
		M. taiwanense strain P2F9704a (AF172443)	96-98							
Methanoculleus	3	*M. chikugoensis* (AB038795)	95-97	•	•	•	•			
		M. palmaeoli (Y16382)	95-97							
		M. marisnigri strain CoCam (AF028693)	95-96							
		M. submarinus (AF531178)	95-96							
Methanosarcinales										
Methanosarcina	9	*M. mazei* strain MT (AY260432)	96-98	•				•	•	•
Methanomethylovorans	8	*M. hollandica* strain ZB (AY260433)	98						•	•
Methanosaeta (formerly *Methanothrix*)	2	*Methanosaeta* sp. (AJ133791)	99					•		
		M. thermophila (AB071701)	91							
		M. soehngenii (X51423)	90							
Methanolobus	1	*M. taylorii* (U20154)	95						•	
Methanobacteriales										
Methanobacterium	2	*M. formicicum* strain FCam (AF028689)	99	•	•					
		M. subterraneum strain C2BIS (X99045)	98							

[a] Abbreviations used: for, formate; 2-prop, 2-propanol + CO_2; 2-but, 2-butanol + CO_2; ac, acetate; MeOH, methanol; $MeNH_2$, methylamines

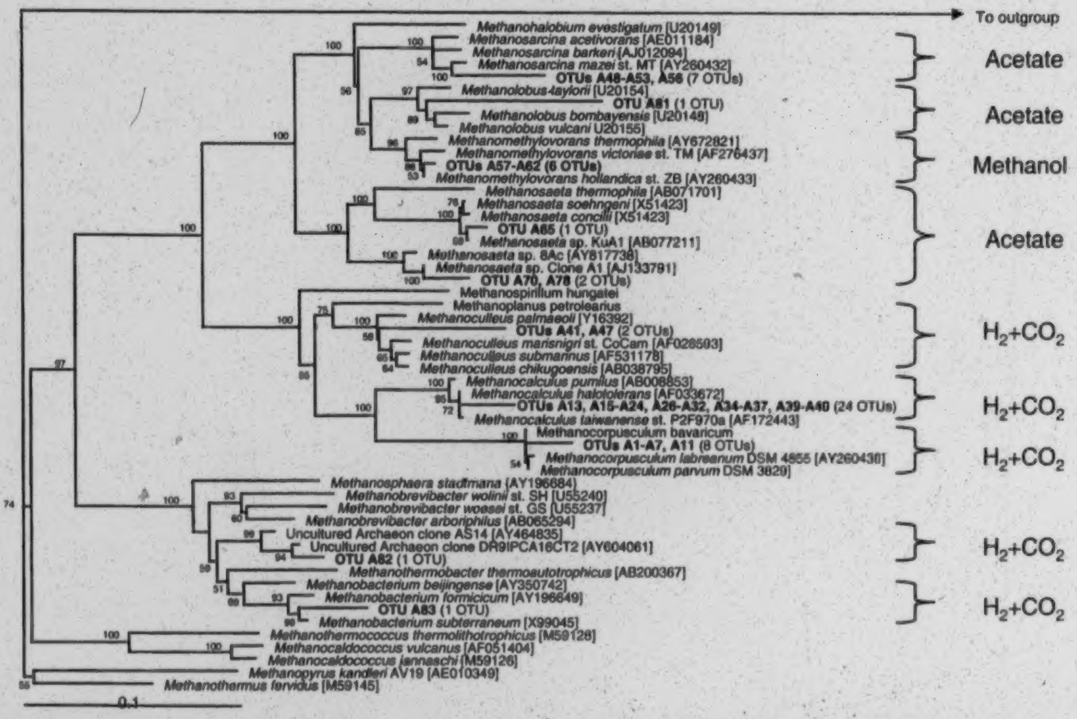

To outgroup
Methanohalobium evestigatum [U20149]
Methanosarcina acetivorans [AE011184]
Methanosarcina barkeri [AJ012094]
Methanosarcina mazei st. MT [AY260432]
OTUs A48-A53, A56 (7 OTUs)
Methanolobus taylorii [U20154]
OTU A81 (1 OTU)
Methanolobus bombayensis [U20148]
Methanolobus vulcani U20155]
Methanomethylovorans thermophila [AY672821]
Methanomethylovorans victoriae st. TM [AF276437]
OTUs A57-A62 (6 OTUs)
Methanomethylovorans hollandica st. ZB [AY260433]
Methanosaeta thermophila [AB071701]
Methanosaeta soehngenii [X51423]
Methanosaeta concilii [X51423]
OTU A65 (1 OTU)
Methanosaeta sp. KuA1 [AB077211]
Methanosaeta sp. 8Ac [AY817738]
Methanosaeta sp. Clone A1 [AJ133791]
OTU A70, A78 (2 OTUs)
Methanospirillum hungatei
Methanoplanus petrolearius
Methanoculleus palmaeoli [Y16392]
OTUs A41, A47 (2 OTUs)
Methanoculleus marisnigri st. CoCam [AF028693]
Methanoculleus submarinus [AF531178]
Methanoculleus chikugoensis [AB038795]
Methanocalculus pumilus [AB008853]
Methanocalculus halotolerans [AF033672]
OTUs A13, A15-A24, A26-A32, A34-A37, A39-A40 (24 OTUs)
Methanocalculus taiwanense st. P2F970a [AF172443]
Methanocorpusculum bavaricum
OTUs A1-A7, A11 (8 OTUs)
Methanocorpusculum labreanum DSM 4855 [AY260436]
Methanocorpusculum parvum DSM 3829]
Methanosphaera stadtmana [AY196684]
Methanobrevibacter wolinii st. SH [U55240]
Methanobrevibacter woesei st. GS [U55237]
Methanobrevibacter arboriphilus [AB065294]
Uncultured Archaeon clone AS14 [AY464835]
Uncultured Archaeon clone DR9IPCA16CT2 [AY604061]
OTU A82 (1 OTU)
Methanothermobacter thermoautotrophicus [AB200367]
Methanobacterium beijingense [AY350742]
Methanobacterium formicicum [AY196649]
OTU A83 (1 OTU)
Methanobacterium subterraneum [X99045]
Methanothermococcus thermolithotrophicus [M59128]
Methanocaldococcus vulcanus [AF051404]
Methanocaldococcus jannaschi [M59126]
Methanopyrus kandleri AV19 [AE010349]
Methanothermus fervidus [M59145]
0.1
Acetate
Acetate
Methanol
Acetate
H_2+CO_2
H_2+CO_2
H_2+CO_2
H_2+CO_2
H_2+CO_2

Figure 3-5: Phylogenetic tree constructed for 16S rRNA libraries derived from acetate-amended and unamended columns, showing the relationship of Archaeal clones (boldface) to known methanogenic species. Clones fall into clusters indicated on the tree by one representative gene. Major substrates of each group of methanogens are shown to the right of the tree. The tree was constructed from partial (~1000 bp) 16S rRNA sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequence difference. The tree was rooted using *E. coli* as an out-group.

As shown in Figure 3-6, nearly all of the clones from the highest dilution tubes from both the acetate-amended and unamended columns contained sequences related to *Methanocalculus* species. *Methanocalculus* spp. are able to use formate and H_2+CO_2 as substrates for methanogenesis, but not acetate. This indicates that *Methanocalculus* spp. are likely present in very high numbers in the MFT in the columns, and therefore are not diluted out during inoculation of the MPN tubes.

The clone libraries constructed from the lowest dilution MPN tubes presumably better reflect the true diversity of methanogenic sequences, but solid conclusions cannot be made regarding the relative proportion of each species in the original sample, because of the biases previously discussed. On the other hand, if the clone libraries indicate that a certain species increases over time with acetate amendment, it may mean that the original tailings inoculum contained a relatively higher proportion of this species. Therefore, a tentative conclusion can be made based on the changes in microbial populations with or without acetate amendment, keeping in mind the culture bias that occurred during incubation in the MPN culture tubes.

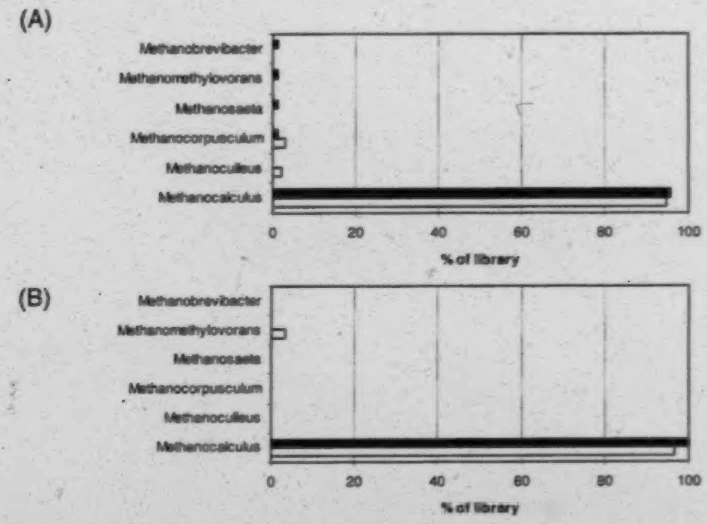


Figure 3-6: Methanogenic genera found in 16S rRNA gene libraries made from the highest dilution MPN tubes inoculated with MFT from acetate-amended and unamended columns, presumably representing dominant methanogenic genera at t=0 (black bars) and t=end (grey bars). A) Column 7 (unamended) and B) Column 9 (amended).

Figure 3-7 shows the changes in metabolic types present in both columns at both times for the lowest dilution MPN tubes. In all libraries, the number of sequences related to methylotrophic *Methanomethylovorans* spp. remains relatively constant, indicating that these species are not affected by the addition of acetate, or by incubation at ambient temperature in the MPN dilution tubes. In both the amended and unamended columns, the numbers of clones related to the acetoclastic *Methanosarcina* spp. increase over time, although at the final time point they still represent only approximately 15% of the clone library. The only major difference in the population shifts between the amended and unamended columns was the increase in clones related to *Methanocorpusculum* spp. that occurs only in the acetate-amended columns. *Methanocorpusculum* spp. are mesophilic methanogens which are able to use H_2+CO_2, formate and 2-propanol as substrates for methanogenesis, however they do not use acetate. This further supports the hypothesis that the Bacterial populations may be supporting the methanogenic populations in the MPN tubes by consuming the added acetate and producing other substrates for the methanogens. It also suggests that *Methanocorpusculum* spp. may be important players in the conversion of acetate to methane, along with a bacterial partner.

With incubation at ambient temperature in the MPN tubes, there was a shift in the methanogenic population from few clones related to acetoclastic species to larger numbers, seen in the samples from the acetate-amended as well as the unamended columns (Figure 3-8). This indicates that the acetoclastic methanogens may have a slight competitive advantage at ambient temperature, which allows their numbers to increase, but are not affected by the increased acetate concentrations. It has been shown that acetoclastic methanogenesis becomes less predominant at high temperature (>25°C) and is out-competed by the syntrophic acetate oxidation and hydrogenotrophic methanogenesis (Schink, 2002), so it does not make sense that the number of clones related to acetoclastic methanogens should increase over time with incubation at room temperature. Possibly there are other factors involved, such as an increased competitiveness of acetoclastic methanogens as the acetate concentrations become depleted over time. Microbial interactions in communities are complex, and it is often difficult to predict which species will dominate in a competitive situation.

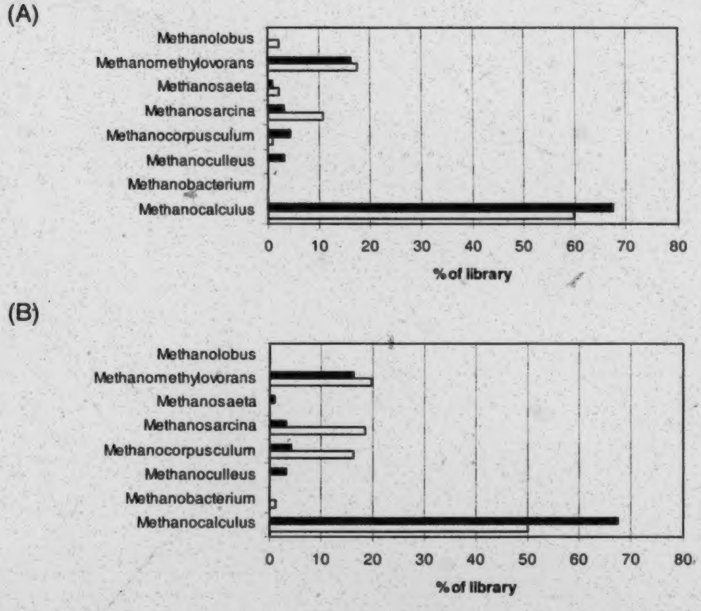


Figure 3-7: Methanogenic genera found in 16S rRNA gene libraries made from the lowest dilution MPN tubes inoculated with MFT acetate-amended and unamended columns, presumably representing the true diversity of methanogenic genera at t=0 (black bars) and t=end (grey bars). A) Column 7 (unamended) and B) Column 9 (amended).

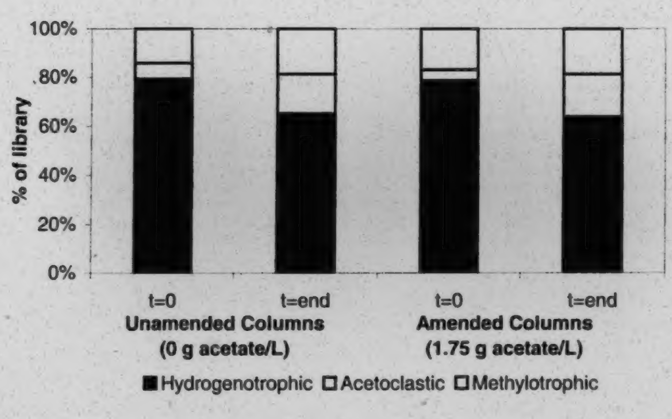


Figure 3-8: Methanogenic species with similar metabolic capabilities are grouped together, from the lowest dilution MPN tubes. This shows possible changes in the mode of methane generation in the MFT.

The results from the analysis of methanogenic species in the columns show the obvious dominance of H_2-consuming methanogens in the MPN culture tubes inoculated with the MFT from acetate-amended and unamended columns. The dominance of hydrogenotrophic methanogens even when the MFT were amended with acetate suggests that a syntrophic interaction between acetate-oxidizing bacteria and hydrogenotrophic methanogens may be occurring in these cultures. The only methanogenic microorganisms that increased in numbers after incubation with acetate were the H_2-consuming *Methanocorpusculum* spp., indicating that these methanogens may be important in the syntrophic oxidation of acetate coupled with hydrogenotrophic methanogenesis in MPN culture tubes from laboratory-incubated samples. Analysis of the Bacterial clones may confirm the presence of syntrophic microorganisms to support this hypothesis. This hypothesis was tested by analysis of the Bacterial clones (Section 3.2.2).

3.2.2 Bacterial Clone Library Results

A very high diversity of microorganisms was initially observed in the Bacterial clone libraries, as virtually every ARDRA pattern was unique. This subsequently influenced further experimental analysis. Bacterial clone libraries were made and analyzed only from the highest methane-positive dilution tubes and only clones that appeared two or more times in a library were sequenced. This degree of diversity was surprising because it was assumed that MFT had a limited range of carbon sources and would not be able to sustain a large number of species.

The same potential problems were encountered with the interpretation of the Bacterial 16S rRNA libraries as with the Archaeal libraries. Those species able to grow best in the methanogenic medium may show up in higher proportions in the clone libraries, and may not faithfully represent the communities present in the original MFT samples. However, the Bacterial species that are present in the highest methane-positive tubes should be the dominant species in the original samples, as less dominant species will have been diluted to extinction.

A large number of OTUs were observed in the Bacterial libraries, even though they were constructed from the highest positive dilution tubes (Figure 3-9).

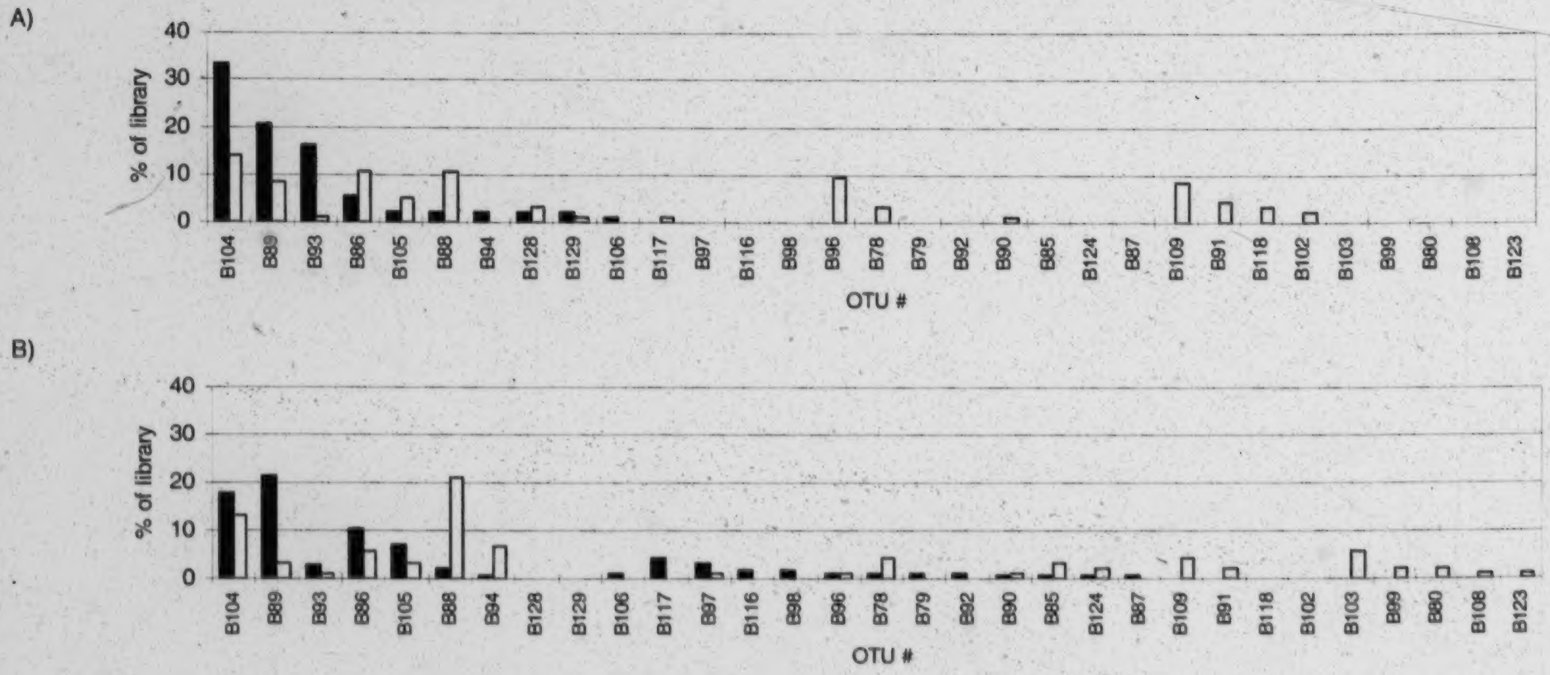


Figure 3-9: Changes in the frequency of patterns occurring over time in Bacterial clone libraries constructed from MPN cultures inoculated with MFT from acetate-amended or unamended columns. Black bars indicate t=0 samples and grey bars indicate t=end samples. A) Column 7 (0 g acetate/ L MFT). B) Column 9 (1.75 g acetate/ L MFT). Only patterns with two or more representatives in any library are shown.

The same general trends were observed with the OTUs from libraries constructed from both the acetate-amended and unamended columns. Clones representing OTUs B89, B93 and B104 all decreased over time, regardless of acetate amendment, indicating that these patterns may represent species which are less able to compete in the incubation conditions (Figure 3-9).

The number of clones in OTU B88 increased with time, regardless of acetate amendment, indicating that these microorganisms may be better able to grow in the incubation conditions provided, and therefore increase in number. Changes were seen in the proportion of clones represented by OTU B86 which increased over time without acetate amendment and decreased in the presence of acetate. This may indicate that these clones represent microorganisms which do not use acetate and are out-competed by microorganisms better able to acetate. Both of these OTUs are related to the genus *Clostridium* (Table 3-5), so there is no real explanation why one may be stimulated by acetate and one may be out-competed when acetate is present.

One representative clone from each pattern appearing two or more times in any library was selected for sequencing. Each sequence was compared to GenBank and RDP II for identification of closely related sequences (Table 3-5).

Table 3-5: Frequency of detection of Bacterial OTUs in clone libraries constructed from the highest dilution MPN culture tubes inoculated with MFT taken from acetate-amended or unamended columns.

		Column 7 (no acetate)		Column 9 (acetate)			Similarity	
	OTU#	t=0	t=end	t=0	t=end	Closest Related Species or Clone [b]	GenBank (%)	RDP II [c]
CFB [a]	B78		9	2	1	uncultured bacterium SHA-107; AJ306739	97	0.786
	B79		3	2	4	uncultured bacterium SHA-107; AJ306739	97	0.789
	B80		2			uncultured bacterium PL-38B1; AY570569	99	0.984
	Total	**0**	**9**	**2**	**1**			
Sphingobacterium	B83				2	uncultured bacterium, ARKCRY2; AY198110	96	0.865
						Hymenobacter sp. MJ1; AF449431	96	–
	Total	**0**	**0**	**0**	**2**			
Clostridia	B85			2		*Clostridium* sp. 9B4; AY554416	95	0.739
	B86	5	10	19	5	*Clostridium* sp. 9B4; AY554416	95	0.744
	B87		1	1	1	*Clostridium* sp. 9B4; AY554416	95	0.760
	B88	2	10	4	19	Uncultured bacterium clone PL-35B7; AY570625	98	0.917
						Clostridium sp. 9B4; AY554416	97	0.822
	B89	19	8	39	3	*Clostridium* sp. 9B4; AY554416	97	0.827
						uncultured bacterium; PL-35B7; AY570625	97	0.910
	B90			2		Uncultured bacterium clone PL-35B7; AY570625	97	0.906
						Clostridium akagii strain CK58; AJ237755	93	–
	B91			1	2	*Clostridium* sp. 9B4; AY554416	96	0.790
						uncultured bacterium; PL-35B7; AY570625	92	0.900
	B92			2		uncultured bacterium; PL-35B7; AY570625	98	0.909
						Clostridium sp. 9B4; AY554416	97	0.814
	B93	15	1	5	1	*Clostridium* sp. 9B4; AY554416	96	0.796
	B94	2		1	6	Uncultured bacterium PL-35-B7; AY570625	98	0.902
						Clostridium sp 9B4; AY554416	96	–
	B95				1	Uncultured bacterium PL-35-B7; AY570625	97	0.909
						Clostridium sp 9B4; AY554416	97	0.817
	B96			3		Clostridium aminobutyricum DSM 2634; X76161	96	0.863
						Uncultured bacterium Eub No. 20; AF395430	97	0.863
	B97		1	8		*Clostridium aminobutyricum* strain DSM 2634; X76161	95	0.788
						Anaerovorax odorimutans strain NorPut; AJ251215	93	0.722
	B98			3		Uncultured bacterium Eub No. 20; AF395430	96	0.829
						Clostridium aminobutyricum DSM 2634; X76161	95	0.827
	B99		3			*Clostridium aminobutyricum* DSM 2634; X76161	95	0.818
						Uncultured bacterium Eub No. 20; AF395430	98	0.913

Continued on next page

		Column 7 (no acetate)		Column 9 (acetate)			Similarity	
	OTU#	t=0	t=end	t=0	t=end	Closest Related Species or Clone [b]	GenBank (%)	RDP II [c]
	B102		8		4	uncultured bacterium PL-5B1; AY570633	97	0.895
						Fusibacter paucivorans; AF050099	94	0.737
	B103		4		2	uncultured bacterium PL-5B1; AY570633	97	0.902
						Fusibacter paucivorans; AF050099	94	0.749
	B104	31	13	32	12	*Soehngenia saccharolytica;* AY353956	97	0.894
	B105	2	5	13	3	*Soehngenia saccharolytica;* AY353956	94	0.585
	B106	2				*Tissierella praeacuta* type strain NCTC 11158; X80832		0.765
	B107				1	*Soehngenia saccharolytica* BOR-Y; AY353956	94	–
	B108				5	*Acetobacterium halotolerans* SyrA5; AY744449	97	0.929
	B109			1	3	*Acetobacterium halotolerans* SyrA5; AY744449	96	0.913
	B116			6	1	Uncultured firmicute Clone P. palm C/A 51; AJ441230	94	0.627
						Clostridium tetanomorphum (NCIMB11547); X68184	87	–
	B117	1		2		*Carnobacterium alterfunditum;* L08623	99	0.968
	B118			1		*Carnobacterium alterfunditum;* L08623	99	0.954
	Total	**79**	**69**	**147**	**75**			
Acholeplasma	B123				2	Uncultured bacterium KD1-22; AY218558	–	0.775
						Uncultured bacterium clone KD4-26; AY188312	96	–
	B124			2		*Acholeplasma axanthum;* AF412968	93	0.633
	Total	**0**	**0**	**2**	**2**			
E. coli	B128	2	3			*E. coli* K12 (U18997)	99	0.960
	B129	2	1			*E. coli* K12 (U18997)	99	0.932
	Total	**4**	**4**	**0**	**0**			
Not Sequenced	**Total**	**10**	**12**	**36**	**12**			
Clone Library Size	**93**	**94**	**187**	**90**				

[a] CFB, Cytophaga-Flavobacterium-Bacteroides division

[b] when closest match was an uncultured clone, the closest cultured species is also shown (if >90% similar).

[c] Ribosomal Database Project II, http://rdp.cme.msu.edu/index.jsp; value is similarity index (S_{ab}), representing the similarity between the two sequences

The majority of Bacterial clones (70 to 80%) belonged to OTUs affiliated with a large, diverse group of *Bacteria* called the clostridia (Table 3-5). These include such genera as *Clostridium*, *Soehngenia*, *Tissierella*, *Anaerobacter*, and *Acetobacterium*, among others. *Clostridium* spp. are Gram positive, endospore-forming, obligatory anaerobic bacteria, which are unable carry out dissimilatory sulfate reduction. In natural environments, these microorganisms carry out the fermentation of organic molecules to acids, alcohols, H_2 and CO_2 (Hippe et al., 1999). These smaller organic molecules and CO_2 can then be used directly by methanogens to produce methane or through a syntrophic relationship involving the oxidation of acetate by a Bacterial species and subsequent methane formation by a hydrogenotrophic methanogen, as described in Section 1.2.1. Other members of the clostridia are homoacetogens, which can grow fermentatively producing only acetate but no H_2, and can also grow autotrophically by the reduction of CO_2 with H_2 to form acetate. As explained in Section 1.2.1, some homoacetogens are able to reverse the acetate-forming reaction and oxidize acetate to produce CO_2 and H_2. Therefore, homoacetogens are the most likely candidates to be involved in the syntrophic oxidation of acetate with the help of a H_2-consuming methanogen (Schink and Stams, 2002).

The clostridia are such a large group of bacteria due to the fact that historically, newly isolated microorganisms which were found to be obligatory anaerobic endospore-formers were placed in the genus *Clostridium*. Over time, this genus became quite large and polyphyletic, so Collins et al. (1994) proposed "clusters" within the clostridia based on phylogenetic relationships. As shown in Figure 3-10, all the clostridial clones from the Bacterial clone libraries fall into four of these clusters, all of which contain species which are known homoacetogens; therefore it is possible that the sequences represented by the clones could also be homoacetogens.

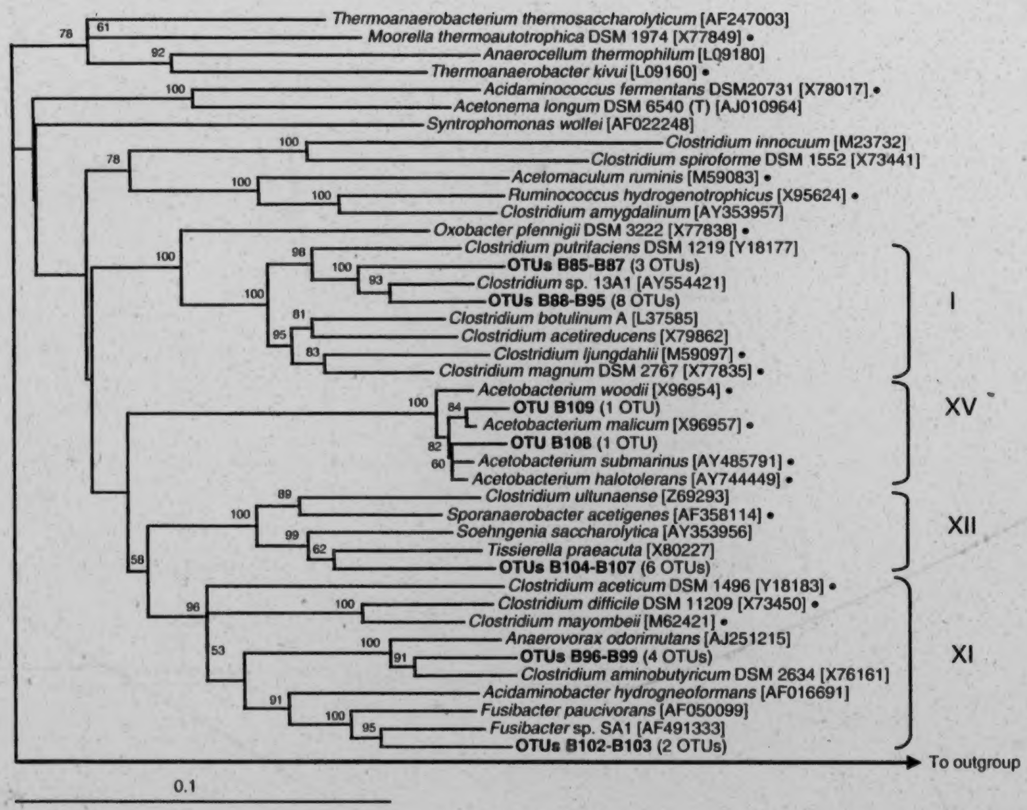

78
61
Thermoanaerobacterium thermosaccharolyticum [AF247003]
Moorella thermoautotrophica DSM 1974 [X77849] •
92
Anaerocellum thermophilum [L09180]
Thermoanaerobacter kivui [L09160] •
100
Acidaminococcus fermentans DSM20731 [X78017] •
Acetonema longum DSM 6540 (T) [AJ010964]
Syntrophomonas wolfei [AF022248]
100
Clostridium innocuum [M23732]
78
Clostridium spiroforme DSM 1552 [X73441]
100
Acetomaculum ruminis [M59083] •
100
Ruminococcus hydrogenotrophicus [X95624] •
Clostridium amygdalinum [AY353957]
Oxobacter pfennigii DSM 3222 [X77838] •
100
98
Clostridium putrifaciens DSM 1219 [Y18177]
100
OTUs B85-B87 (3 OTUs)
93
Clostridium sp. 13A1 [AY554421]
100
OTUs B88-B95 (8 OTUs)
81
Clostridium botulinum A [L37585]
95
Clostridium acetireducens [X79862]
83
Clostridium ljungdahlii [M59097] •
Clostridium magnum DSM 2767 [X77835] •
I
Acetobacterium woodii [X96954] •
100
84
OTU B109 (1 OTU)
Acetobacterium malicum [X96957] •
82
OTU B108 (1 OTU)
60
Acetobacterium submarinus [AY485791] •
Acetobacterium halotolerans [AY744449] •
XV
89
Clostridium ultunaense [Z69293]
100
Sporanaerobacter acetigenes [AF358114] •
99
Soehngenia saccharolytica [AY353956]
62
Tissierella praeacuta [X80227]
58
OTUs B104-B107 (6 OTUs)
XII
Clostridium aceticum DSM 1496 [Y18183] •
96
100
Clostridium difficile DSM 11209 [X73450] •
Clostridium mayombeii [M62421] •
100
Anaerovorax odorimutans [AJ251215]
53
91
OTUs B96-B99 (4 OTUs)
Clostridium aminobutyricum DSM 2634 [X76161]
91
Acidaminobacter hydrogneoformans [AF016691]
100
Fusibacter paucivorans [AF050099]
95
Fusibacter sp. SA1 [AF491333]
OTUs B102-B103 (2 OTUs)
XI
To outgroup
0.1

Figure 3-10: Phylogenetic tree constructed from 16S rRNA gene sequences showing the relationship of some Bacterial clones to known clostridia species. Clones fell into groups, represented on the tree by one representative sequence. Known homoacetogenic or syntrophic microorganisms are indicated by the symbol •. Clusters (I, XI, XII and XV) are as proposed by Collins et al. (1994). Tree was calculated using partial (~1500 bp) 16S rRNA gene sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replications, and are indicated by the numbers on each node. Bootstrap values below 50% are not shown. The scale bar represents 10% sequence difference. The tree was rooted using *Methanosarcina mazei* as an outgroup.

The distribution of the clones related to the different clusters of the clostridia changes with time (Figure 3-11). The proportion of clones falling into Cluster I, which contains many of the true *Clostridium* spp., remains relatively constant over time with or without acetate amendment. Cluster XI clones increase with time without acetate amendment, and decrease with time when acetate is added. The closest related sequences in this cluster were *Clostridium aminobutyricum*, and a *Fusibacter* sp., neither of which uses acetate as a substrate (Hippe et al., 1999) and therefore may be out-competed when acetate-utilizers are stimulated by the addition of acetate. Cluster XII clones decrease with time both with and without acetate amendment. This cluster contains fermentative microorganisms such as *Soehngenia saccharolytica* and *Tissierella praeacuta*, which are involved in the degradation of organic matter in anaerobic environments (Hippe et al., 1999). It is not clear why these appear to be out-competed and decrease in proportion with incubation in a laboratory setting.

Clones in Cluster XV, which are closely related to *Acetobacterium* spp., appear only in the library made from the acetate-amended column at the end of the incubation period. The genus *Acetobacterium* consists only of homoacetogenic bacteria, which may be able to reverse the acetate-forming reaction and oxidize acetate with the help of a hydrogen-consuming methanogen. Because these microorganisms appear only after incubation with acetate, this could indicate they are involved in a syntrophic relationship in the degradation of acetate in the methanogenic cultures, and are stimulated by the addition of acetate.

In addition to the clones related to members of the clostridia, sequences related to other microorganisms were observed, including those distantly related to the genera *Hymenobacter* and *Cytophaga*, in the family Cytophagales. *Hymenobacter* is a newly described genus in the *Cytophaga-Flavobacterium-Bacteroidetes* (CFB) group. These bacteria are strictly aerobic (Oren, 2004), and their discovery in the highly anaerobic methanogenic culture is strange. Members of the family Cytophagales are ubiquitous bacteria found in many types of aerobic and anaerobic environments, including soil and seawater. They are organotrophs and play a major role in the degradation of organic matter in anaerobic, microaerophilic, and aerobic environments (Reichenbach, 1999a), and may be involved in the degradation of organic molecules in the MFT.

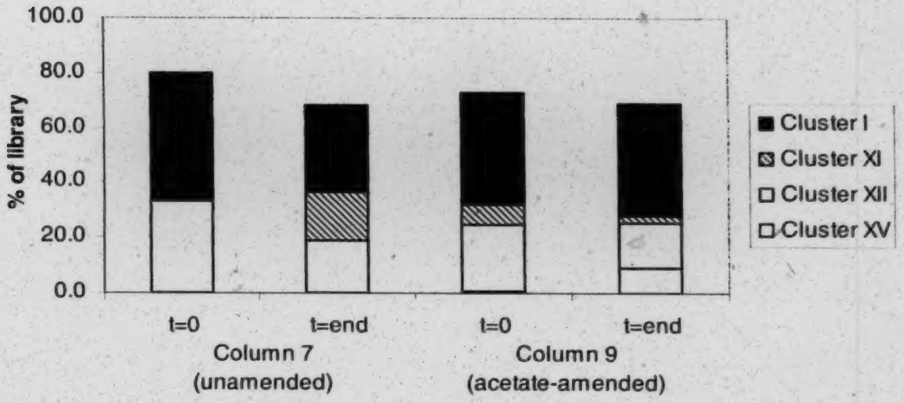


Figure 3-11: Changes in the proportion of clostridial clones derived from MFT after incubation with and without acetate amendment. Clones are grouped into clusters as proposed by Collins et al. (1994), indicated on Figure 3-10.

Also, sequences related to a member of the genus *Carnobacterium* were found. *Carnobacterium* spp. are related to the clostridia and the genus *Lactobacillus* and are sometimes involved in food spoilage. They have also recently been discovered in Antarctic lake sediments Hammes, 2004 #229}. They are facultatively anaerobic and heterofermentive, and probably play a role in the fermentation of organic matter in the MFT. They can also produce formate as a byproduct, which can be used directly by methanogens for methane production (Hammes and Christian, 2004).

One sequence related to *Acholeplasma* spp. was found. *Acholeplasma* is a type of mycoplasma, a genus lacking cell walls. They are facultative aerobes, so they could survive in the anaerobic tailings environment (Madigan et al., 2000), although their role in the production of methane in the MFT is not clear.

3.2.3 Conclusions from clone libraries constructed from the columns

Archaeal 16S rRNA gene sequence analysis indicates that hydrogenotrophic methanogens are the dominant methanogenic species in MPN culture tubes from all MFT samples analyzed, even though some of the samples analyzed were amended with acetate. Bacterial 16S rRNA gene sequence analysis revealed the presence of large numbers of clostridia, some of which are related to known homoacetogens. Some homoacetogens may be able to reverse the acetate-forming reaction, and oxidize acetate to CO_2 with the help of a hydrogen-consuming methanogen, as explained in Section 1.2.1. These discoveries support the hypothesis that methane may be produced in the cultured MFT via a syntrophic relationship between acetate-oxidizing homoacetogens and H_2-consuming methanogens. Although this may not be the pathway of methane production *in situ*, it does provide information about how the methanogenic consortia are metabolically flexible.

3.2.4 Clone library coverage and species richness

Accumulation curves were constructed for all of the Archaeal and Bacterial libraries constructed from MPN culture tubes inoculated with acetate-amended or unamended MFT. The accumulation curves show that a sufficient number of clones was sampled to represent the diversity in the Archaeal clone libraries, as the accumulation curves for libraries constructed from the lowest dilution tubes appear to approach a horizontal line (Figure 3-12). The accumulation curves for the Archaeal libraries constructed from the highest positive MPN dilution tubes are similar to those constructed from the lowest dilution tube and are asymptotic (not shown).

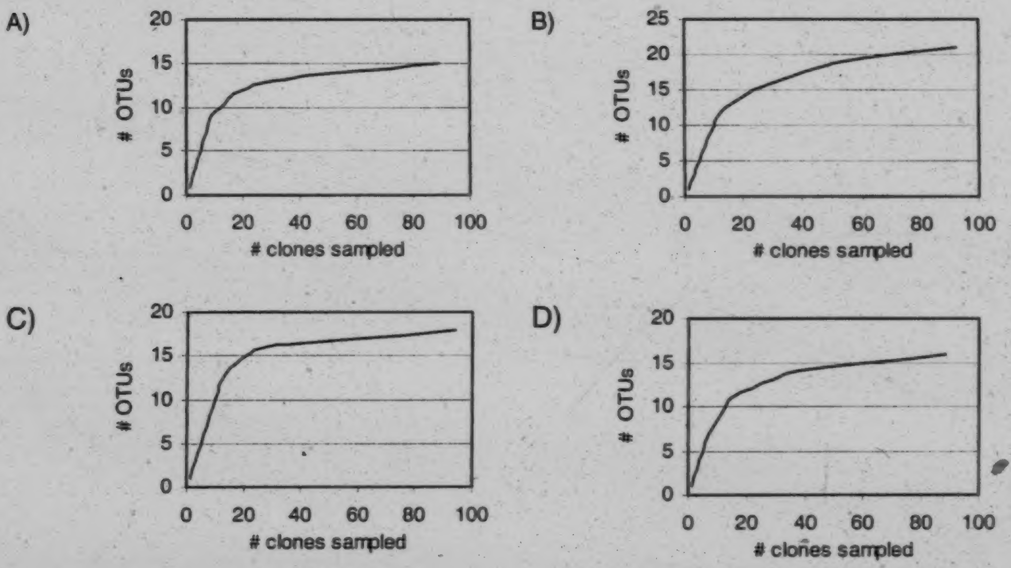


Figure 3-12: Accumulation curves for Archaeal libraries constructed from the lowest dilution MPN culture tube inoculated with MFT from acetate-amended or unamended columns at t=0 and t=end. A) Column 7 (unamended) t=0; B) Column 7 (unamended) t=end; C) Column 9 (acetate-amended) t=0; D) Column 9 (acetate-amended) t=end.

Accumulation curves for the four Bacterial clone libraries constructed from the highest positive MPN dilution tubes are slightly different (Figure 3-13), and may indicate that more clones should have been sampled to fully represent the diversity present in these cultures. Approximately twice as many clones were sampled for the Column 9 t=0 library as for the other libraries, and the accumulation curve for this library does approach a horizontal line (Figure 3-13C). This shows that sampling more clones provides a more accurate representation, as compared to the curve for Column 7 t=end, where the curve is only beginning to plateau (Figure 3-13B). However, sufficient clones were probably sampled to get an initial idea of the diversity present, since OTUs representing similar patterns tended to be phylogenetically related (e.g. Table 3-5).

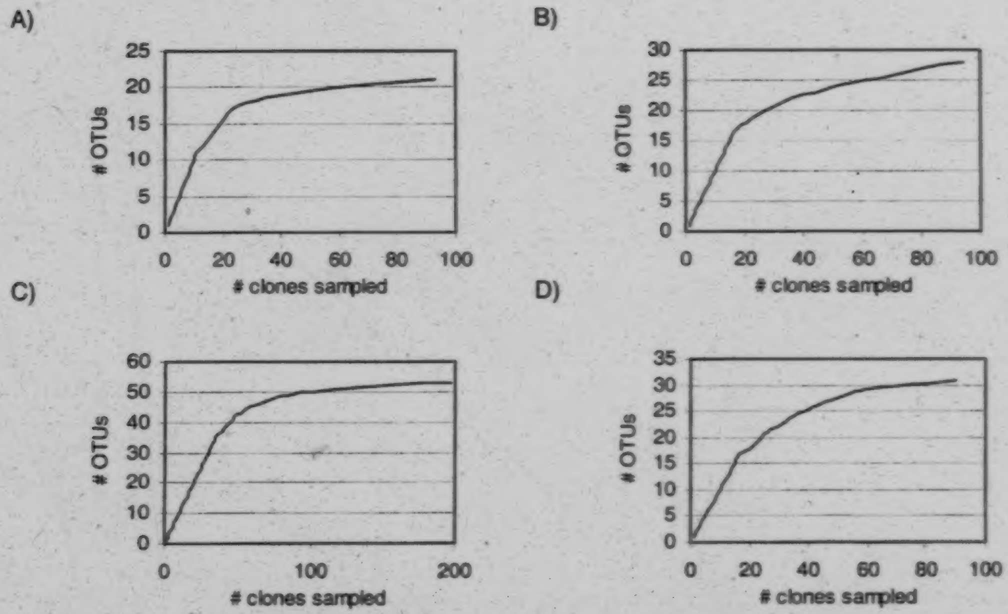


Figure 3-13: Accumulation curves for Bacterial libraries constructed from the lowest dilution MPN culture tube inoculated with MFT from acetate-amended or unamended columns at t=0 and t=end. A) Column 7 (unamended) t=0; B) Column 7 (unamended) t=end; C) Column 9 (acetate-amended) t=0; D) Column 9 (acetate-amended) t=end.

Coverage calculations also indicate that more Bacterial clones should have been sampled to more accurately represent the diversity present in the samples (Table 3-6). The Archaeal libraries generally had higher coverage, indicating lower Archaeal diversity present compared to Bacterial diversity. Also, the libraries constructed from the lowest MPN dilution tubes had a generally lower coverage than those constructed from the highest positive MPN dilution tubes, indicating that the lowest dilution tubes did indeed contain a higher diversity of sequences compared to the lower dilution tubes, although the diversity was probably accurately represented by the size of the Archaeal clone libraries. All of the Bacterial libraries had coverage of less than 0.90, with three having coverage of close to 0.80, indicating that these libraries may not have been big enough to accurately represent the Bacterial diversity present.

Shannon-Weaver indices, representing the species richness of the samples, were also calculated. The higher richness values for the Bacterial libraries correlate with the lower coverage of these libraries, indicating further that more clones should have been sampled to represent the higher diversity of these samples. The Archaeal 16S rRNA gene libraries constructed from the highest dilution MPN tubes had higher species richness values, also supporting the presumption that these culture tubes will represent the diversity present in the original MFT samples, whereas the less diverse lowest dilution tubes only have a few, dominant species.

Table 3-6: Coverage calculations for Archaeal and Bacterial libraries constructed from MPN culture tubes inoculated with MFT from acetate-amended or unamended columns. Coverage was calculated using Good's coverage calculation (Equation 1-6) (Good, 1953). Species richness was calculated using the Shannon-Weaver index (Equation 1-8)(Shannon and Weaver, 1949).

	Coverage		Species Richness	
Archaeal Clone Libraries	lowest dilution	highest dilution	lowest dilution	highest dilution
Column 7 t=0	0.90	0.92	1.77	1.06
Column 7 t=end	0.88	0.96	2.43	1.29
Column 9 t=0	0.87	0.94	1.84	1.10
Column 9 t=end	0.92	0.98	1.98	1.06
Bacterial Clone Libraries				
Column 7 t=0	–[a]	0.90	–	2.17
Column 7 t=end	–	0.82	–	2.87
Column 9 t=0	–	0.81	–	2.99
Column 9 t=end	–	0.82	–	2.95

[a] Bacterial clone libraries were not constructed for the lowest dilution MPN culture tubes

3.3 Analysis of neat MFT collected from active settling basins

The studies described in Sections 3.1 and 3.2 were based on MFT that had been cultured in a laboratory setting. They were first incubated in columns, with or without acetate-amendment, and then were inoculated into MPN culture medium and incubated again. These manipulations will certainly have caused shifts in the microbial community structure, favoring those microorganisms which are best able to grow and survive in the laboratory conditions. To obtain a more realistic view of the *in situ* microbial communities, uncultured MFT were analyzed using the same methods.

MFT samples were collected from MLSB in May 2004 and WIP in April 2004. The relevant chemical and physical characteristics were determined by Dr. M. MacKinnon of Syncrude (Table 3-7, Appendix 7).

In MLSB, the tailings have become stratified, with older tailings at the bottom of the solids layer, and new tailings deposited on top. The stratification provides different environments for the microorganisms present in the tailings. The deeper, older tailings will be depleted in available carbon substrates and electron acceptors, whereas the newer tailings at shallower depths will have more available nutrients. This can be seen in the gradients of various electron acceptors and nutrients, as well as a temperature gradient (Table 3-7). WIP, however, contains tailings originally pumped from MLSB, so this stratification does not occur. Nutrient and carbon source concentrations do not vary as considerably with depth as seen in MLSB (Table 3-7).

Table 3-7: Relevant properties of MFT samples taken from MLSB and WIP.

	MLSB (sample depth, m)				WIP (sample depth, m)		
	6	10	20	30	6	10	20
pH	7.37	7.00	7.54	7.96	7.75	7.73	7.90
Temperature (°C)	12	16.9	22	21.2	11.2	14.2	15.8
Redox Potential (mV)	-197	-121	-112	-198	-239	-319	-128
Solids content (g/100g)	42.3	43.4	60.2	71.8	32.9	36.4	43.2
Bitumen content (g/100g)	2.57	2.24	1.68	1.11	2.45	2.13	2.36
Naphtha content (wt%)	na*	na	na	na	0.02	0.09	0.04
DOC (mgC/L)	44	44	50	56	53.6	53.2	51.0
Naphthenic Acids (mg/L)	42.3	40.6	84	95	69.0	67.2	69.2
Sulfate (mg/L)	0.1	12.8	19.6	36.0	45	70.1	18.9
HCO_3^- (mg/L)	1700	1790	1220	1470	1320	1400	1300
Nitrate + nitrite (mgN/L)	0.022	0.029	0.036	0.038	0.011	0.011	0.015
Total Iron (mg/L)	0.45	0.61	0.21	0.22	0.07	0.08	0.16

*na=data not available

3.3.1 Methanogen and SRB enumeration

Four samples from increasing depth in MLSB (6, 10, 20 and 30 m) and three samples from WIP (6, 10 and 20 m) were used for MPN analysis for SRB and methanogens.

The SRB MPN enumeration used a modified Butlin's medium. Tubes were scored positive for SRB growth by a black precipitate of FeS formed on an iron nail in each MPN tube. The MPN analysis of methanogens used a bicarbonate medium containing 6.805 g acetate/L and a headspace containing 50% H_2, 15% CO_2, balance N_2. This should allow growth of both acetoclastic and hydrogenotrophic methanogens. The tubes were analyzed for methane production using gas chromatography. After 30 days, the MPN estimates were still very low. After two months the tubes were analyzed again, resulting in numbers that were much higher. These data are presented in Figure 3-14 with sulfate concentrations (provided by Syncrude; see Table 3-7). The trends observed between the numbers of SRB and methanogens are different to those seen in 1998 by Holowenko et al. (2000) (Figure 1-6). In MLSB, the highest number of methanogens occured at 10 and 20 m, and the lowest at 30 m (Figure 3-14A). SRB numbers were fairly constant throughout all the depths, with a slight decrease with increasing depth (Figure 3-14B). I did not observe the same trend that Holowenko et al. (2000) observed in 1998, that as the numbers of SRB decreased with depth, the numbers of methanogens increased. The numbers of SRB also did not decrease with the sulfate concentration. Methanogens in WIP were lowest at the 6 m depth, and higher at 20 and 30 m. SRB also increased with depth. There was a negative correlation between sulfate concentration and SRB, as the highest number of SRB occurred at 20 m, where the sulfate concentration is lowest. This may be because the higher numbers of SRB in the past have depleted the sulfate in the MFT.

The differences in numbers between 1998 and now may indicate that the microbial populations have changed since 1998. The sulfate concentrations in MLFB in 1998 decreased with depth, but now the sulfate concentrations increase with depth. This change in sulfate concentration may be the cause of the changes in methanogen and SRB counts. These differences may also be due to the fact that the MFT that I used were not freshly sampled, but had been stored at 4°C for a number of months. Holowenko et al. (2000) did not add H_2 to the methanogen MPN culture medium, which also may have caused the differences in methanogen numbers in this study.

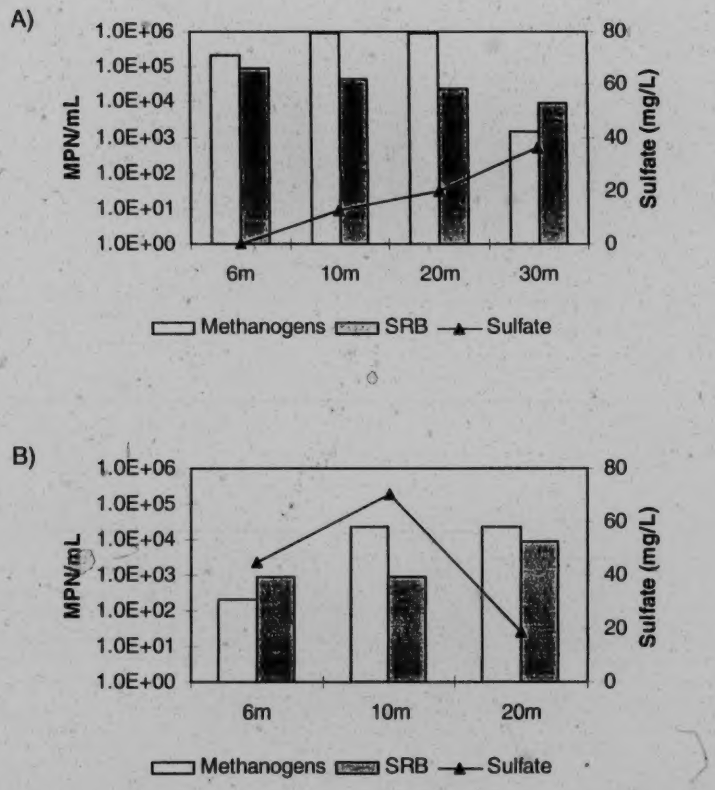


Figure 3-14: A) MPN enumeration of methanogens and SRB in MLSB MFT samples from different depths. B) MPN enumeration of methanogens and SRB in WIP MFT samples from different depths. The sulfate concentration of each sample is also indicated on the graphs by the triangle symbols.

3.3.2 Methane production from the MFT

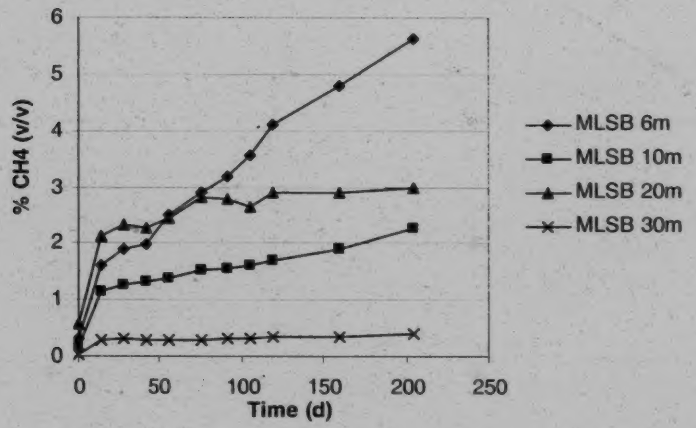
To complement the MPN enumeration of methanogens in the MFT samples, serum bottles containing 50 mL of MFT (one for each depth) were set up and the amount of methane in the headspace was measured every 2 weeks (Figure 3-15). Although six of the seven cultures produced methane after 75 days of incubation, the highest amount of methane detected was less than 6% (v/v) for the MLSB cultures, and less than 0.6% (v/v) for the WIP cultures, which was much less than has been previously observed for other MFT samples. This may be due to the fact that the tailings had been stored at 4°C for several months, or that they were handled or stored improperly (i.e. exposure to air) before they were received. However, DNA was extracted from all of the samples, indicating that the microbes were still present but may no longer be viable.

More methane was produced from the MLSB MFT than the WIP MFT (Figure 3-15). The 6 and 20 m samples from MLSB appear to have produced the most methane, consistent with the high methanogen counts observed for these samples (Figure 3-14). The 10 m MLSB sample, although having one of the highest methanogen counts, produced slightly less methane than two other samples, and production appeared to have leveled off. This may be due to the fact that this particular sample is extremely thick, and any methane that was produced may be trapped in bubbles within the tailings, although the serum bottles were shaken before removing a headspace sample for methane analysis. The 30 m MLSB sample had very few methanogens as indicated by the MPN enumeration (Figure 3-14), and produced much less methane than the samples from the other depths (Figure 3-15A).

There is a relationship between the sulfate concentration of the MFT and the total volume of methane produced. Sulfate concentrations in MLSB increase with depth (Figure 3-14A) and the amount of methane produced decreases with the depth of the sample (Figure 3-15). Sulfate concentrations are much higher in the WIP samples (Figure 3-14A, up to 80 mg/L) and the methane produced from the WIP samples is subsequently much lower. This supports the fact that methanogens are out-competed by SRB when high concentrations of sulfate are present.

In comparison, all of the WIP MFT samples produced very little methane (Figure 3-15B), less than 0.6% (v/v) even for the most active sample (10 m). The 20 m sample appeared to have the most methanogens according to the MPN enumeration, but this culture produced no detectable methane after 200 d of incubation.

A)



B)

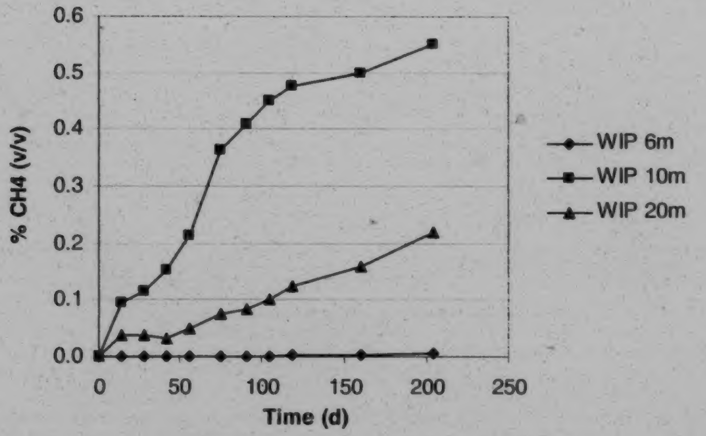


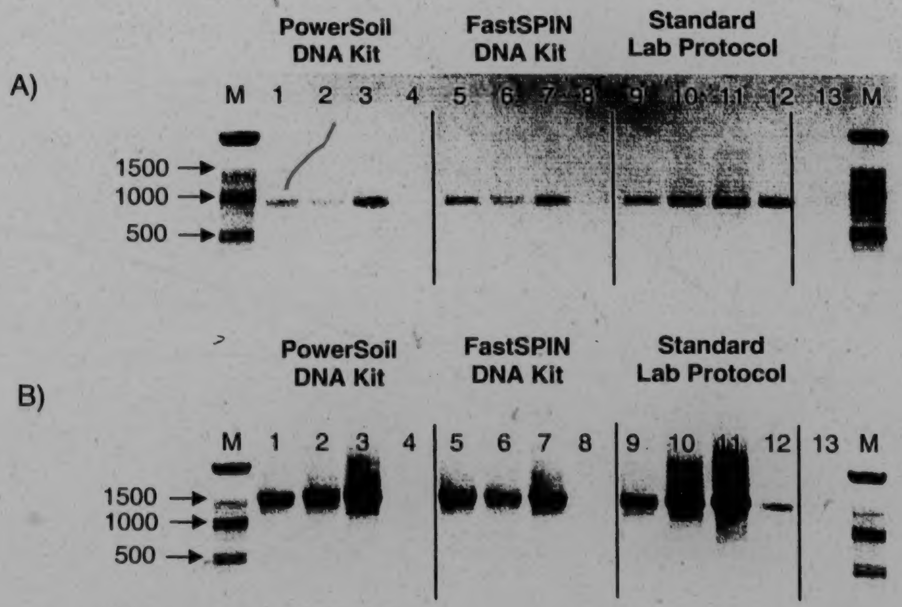
Figure 3-15: Methane produced by unamended MFT samples from different depths incubated in serum bottles. A) MLSB MFT; B) WIP MFT.

3.3.3 Optimization of DNA extraction from the neat MFT

When this project began, I was unsure whether or not it would be possible to efficiently extract genomic DNA from the neat MFT, due to presence of interfering components such as clay particles and residual bitumen. As a consequence, the project began by analyzing cultured MFT, which would allow the methanogenic species to be enriched, and for a large proportion of these componants to be removed prior to DNA extraction. After developing a reliable DNA extraction method using the cultured MFT, extraction from uncultured MFT was attempted.

Three extraction protocols were compared to determine which would provide the highest quality DNA for PCR amplification from neat MFT. Also, incubation at 70°C for 10 min was attempted to see if this would achieve better cell lysis and higher DNA yield. One sample containing MFT seeded with MPN culture was processed to determine if DNA could be purified from the tailings, in case the numbers of microorganisms in the tailings was too low to be detected by PCR amplification.

Results from the first experiment using the small beadbeater (Model 221.BX, Biospec Products) showed that all three methods were suitable for lysing Bacterial cells (Figure 3-16) introduced into the neat MFT. However, when the DNA was used as a template for amplification of Archaeal 16S rRNA genes, our standard method clearly provided the best template. The amplification products from DNA obtained from the two DNA extraction kits had a product band of the correct molecular weight in the positive control, and fainter bands for the tailings samples seeded with MPN culture. However, the standard laboratory protocol provided the brightest band for both the positive control and the heat-treated samples. Heat treatment did not appear to provide more DNA when either of the kits was used, possibly due to degradation of the DNA or increasing the solubility of hydrocarbon contaminants (the samples which were heated had a darker color during processing, and the dark color carried over further into the purification steps in all the methods tested).



A)
PowerSoil DNA Kit
FastSPIN DNA Kit
Standard Lab Protocol
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1500
1000
500
B)
PowerSoil DNA Kit
FastSPIN DNA Kit
Standard Lab Protocol
M 1 2 3 4 5 6 7 8 9 10 11 12 13 M
1500
1000
500

Figure 3-16: Comparison of various methods for extraction and purification of DNA from neat MFT, resolved by electrophoresis on a 1% agarose gel. Extracted DNA was used as a template for A) Archaeal 16S rRNA gene amplification (expected product ~950 bp)and B) Bacterial 16S rRNA gene amplification (expected product ~1500 bp). Lane M is a commercial molecular weight marker DNA ladder (sizes noted in basepairs). Lanes 1, 5, 9: 0.2 mL tailings + 0.1 mL MPN culture. Lanes 2, 6, 10: 0.2 mL tailings + 0.1 mL MPN culture, 10 min at 70°C. Lanes 3, 7, 11: 0.3 mL MPN culture. Lanes 4, 8, 12: Negative extraction control. Lane 13: Negative PCR control.

In this experiment, the negative control used in our beadbeating protocol was contaminated (lane 12, Figure 3-16), however this was not particularly important because the experimental objective was to find a method that could provide amplifiable DNA from the tailings.

The day after this experiment was conducted we received a new beadbeater machine, the Fast Prep Cell Disrupter (BIO101 Systems). Another extraction experiment was conducted to compare our beadbeating method with the FastSPIN kit when the new beadbeater was used. This extraction included a sample containing only neat MFT, to show that amplifiable DNA could be isolated from the neat samples. The purified DNA was used as a template in a PCR to amplify Archaeal 16S rRNA genes (Figure 3-17). Again, our beadbeating protocol provided a better DNA template than the FastSPIN DNA Extraction kit. Again, heat treatment did not appear to provide a better DNA template.

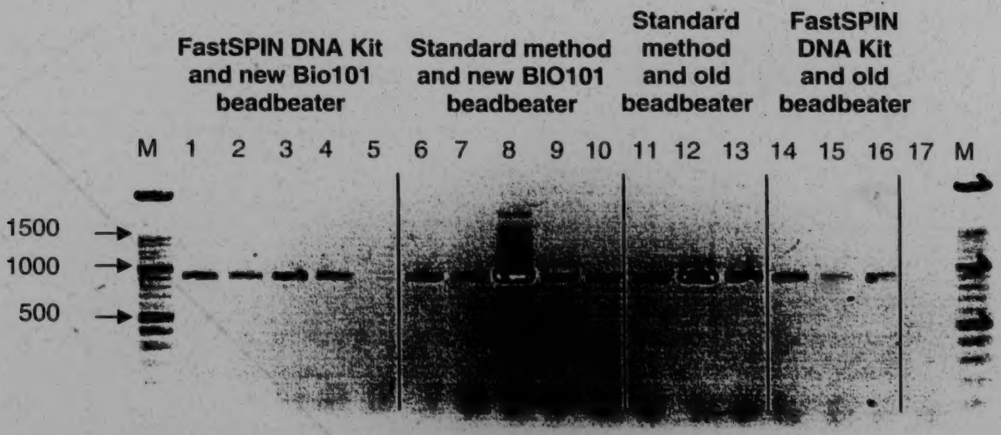

FastSPIN DNA Kit and new Bio101 beadbeater
Standard method and new BIO101 beadbeater
Standard method and old beadbeater
FastSPIN DNA Kit and old beadbeater
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 M
1500
1000
500

Figure 3-17: Comparison of two methods for extraction of DNA from oil sands tailings using the new BIO 101 Systems beadbeater. Extracted DNA was used as a template for PCR amplification of Archaeal 16S rRNA genes (expected product ~950 bp). Lane M is a commercial molecular weight marker (sizes indicated in bp). Lanes 1, 6, 11, 14: 0.2 mL tailings + 0.1 mL MPN culture. Lanes 2, 7, 12, 15: 0.2 mL tailings + 0.1 mL MPN culture, 10 min at 70°C. Lanes 3, 8, 13, 16: 0.3 mL MPN culture. Lanes 4, 9: 0.3 mL tailings. Lanes 5, 10: Negative extraction control. Lane 17: Negative PCR control.

The important result in this experiment was that DNA was obtained from the neat tailings without seeding with MPN culture (lane 4 Figure 3-17). The band was much less intense than the one obtained for the positive control (lane 3), or the tailings seeded with MPN culture (lane 1), however this gel shows that it is possible to obtain enough template DNA from the untreated tailings. The standard lab protocol also provided amplifiable DNA (lane 9), but because the negative control indicates contamination, this does not prove that the DNA in lane 9 is solely from the tailings.

Several speeds (4.5, 5.0, 5.5, 6.0 and 6.5 m/s) were compared using the Bio101 Systems beadbeater to determine which would give the best DNA template. It was determined that a speed of 6.0 m/s gave the highest yield of PCR product using Bacterial-specific primers, with the least possibility of DNA shearing (Figure 3-18). This was the setting that was used for all subsequent DNA extractions from the raw MFT.

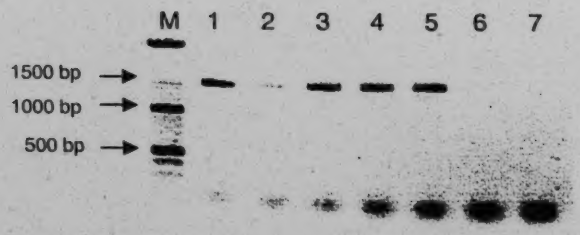


Figure 3-18: Comparison of genomic DNA extraction speeds using the BIO101 Systems bead beater. Extracted DNA was used as a template for Bacterial 16S rRNA genes amplification (expected product ~1500 bp). Lane M is a commercial molecular weight marker DNA ladder (sizes noted in basepairs). Lanes 1-5: 25 µL of PCR product with DNA template obtained from beadbeating at speeds 4.5, 5.0, 5.5, 6.0 and 6.5 m/s, respectively. Lane 6: Negative extraction control. Lane 7: Negative PCR control.

3.3.4 Archaeal species in neat MFT

Using the optimized DNA extraction protocol, new subsamples of MFT were extracted and the DNA was used to construct Archaeal and Bacterial 16S rRNA gene libraries. The results from the Archaeal libraries are presented in this section and the results from the Bacterial libraries are presented in Section 3.3.5.

Archaeal 16S rRNA libraries were constructed from tailings from each depth of MLSB and WIP. Restriction enzyme screening of these clones indicated the presence of 55 different ARDRA patterns (Figure 3-19, Figure 3-20), many of which occurred only once. Two dominant OTUs were observed in all of the libraries (A70 and A71), and their proportions did not appear to change significantly with depth or between the two sampling sites. There were several other minor OTUs observed, but no correlation could be made between the distribution of OTUs and depth or sampling site. No dominant OTUs were observed only at certain depths, or only in one pond and not the other. Each sample does contain some unique OTUs, which occur in very low numbers but do not appear in any of the other libraries.

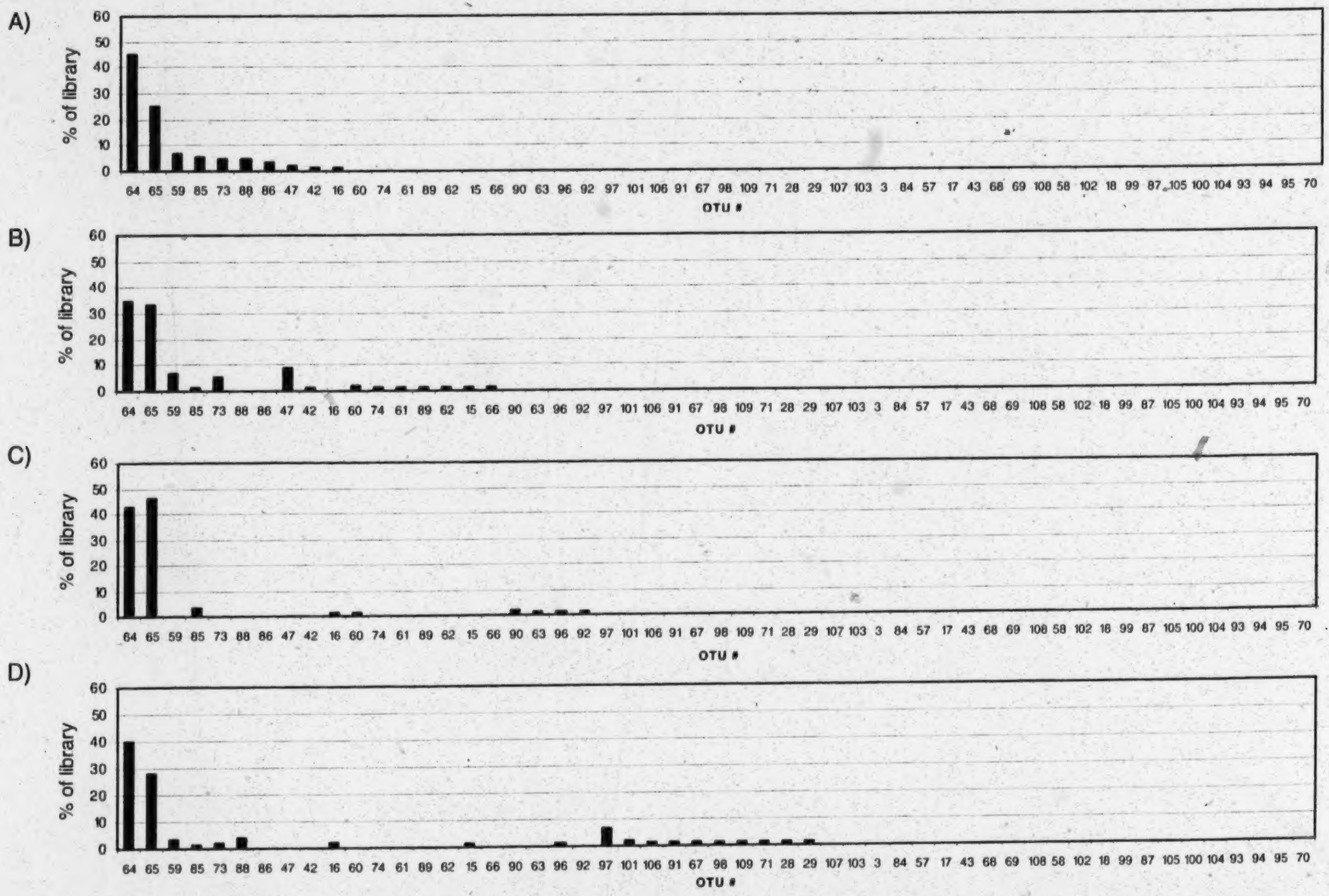


Figure 3-19: Frequency of OTU occurrence in Archaeal 16S rRNA libraries constructed from MLSB MFT. A) MLSB 6 m; B) MLSB 10 m; C) MLSB 20 m; D) MLSB 30 m.

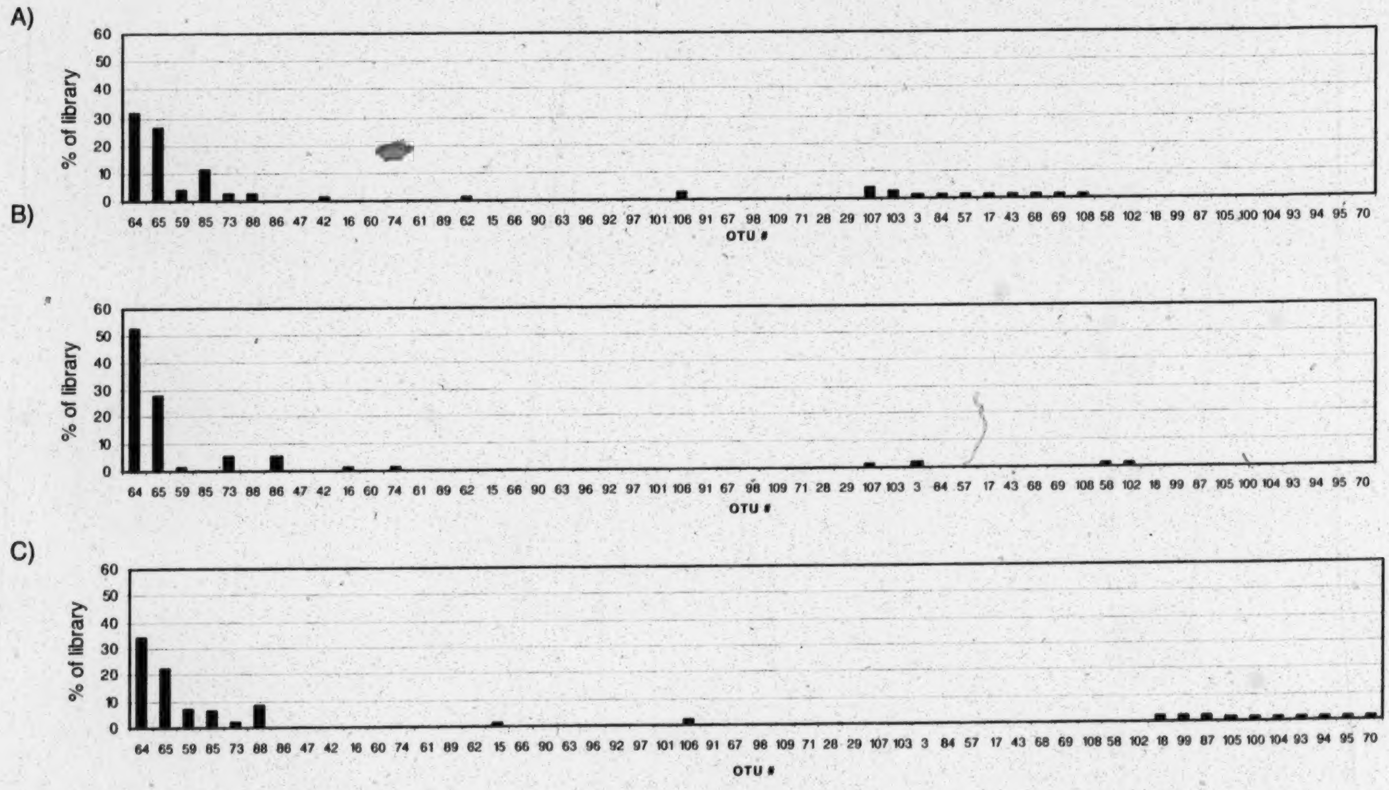


Figure 3-20: Frequency of OTU occurrence in Archaeal 16S rRNA libraries constructed from WIP MFT samples. A) WIP 6 m; B) WIP 10 m; C) WIP 20 m.

Even though there were more OTUs represented in the Archaeal 16S rRNA gene clone libraries than were found in the MPN culture tubes, the Archaeal communities in the tailings appear to be less diverse. The cloned sequences represented only four genera of methanogens (Table 3-8), with a significant number of clones clustering with other uncultured clones in the Crenarchaeota kingdoms (Appendix 3).

Although there were more OTUs in the neat MFT libraries than the acetate-amended and unamended column libraries (Section 3.2), when representative clones were sequenced and identified, the methanogenic populations appeared to be much less diverse. Different OTUs represented minor variations in sequence rather than sequences from different species. The phylogenetic relationships of cloned sequences to known methanogenic microorganisms are shown in Figure 3-21 and Table 3-8. The only known groups to which sequences in the libraries are related are *Methanocorpusculum*, *Methanocalculus*, and *Methanoculleus* which are all hydrogenotrophic methanogens, the methylotrophic *Methanomethylovorans* and the acetoclastic *Methanosaeta*.

The relative proportions of Archaeal clones assigned to each family is shown in Figure 3-22. The obvious dominance of acetoclastic methanogens (Figure 3-23), especially *Methanosaeta* spp. is in sharp contrast to the libraries constructed from MPN tubes inoculated with MFT samples from acetate-amended and unamended columns, where there was a dominance of hydrogenotrophic methanogens, particularly *Methanocalculus* spp (Figure 3-7).

Table 3-8: Archaeal OTUs found in neat MFT from MLSB and WIP. Number of clones in each OTU, % similarity to closest related species or clone, and total number of clones in each taxonomic group are shown.

		Number of clones detected								
		MLSB				WIP				
	OTU	6 m	10 m	20 m	30 m	6 m	10 m	20 m	Closest Related Species or Clone	Similarity (GenBank)
Euryarchaeota										
Methanomicrobiales										
Methanocorpusculum	A3					1	2		*Methanocorpusculum parvum* st. DSM 3829; AY260435	99
Total		**0**	**0**	**0**	**0**	**1**	**2**	**0**		
Methanocalculus	A15		1		1			1	*Methanocalculus pumilus*; AB008853	98
	A16	1		1	2		1		*Methanocalculus pumilus*; AB008853	97
	A17					1			*Methanocalculus pumilus*; AB008853	97
	A18						1	1	*Methanocalculus pumilus*; AB008853	97
	A28				1				*Methanocalculus pumilus*; AB008853	97
	A29				1				*Methanocalculus pumilus*; AB008853	98
Total		**1**	**1**	**1**	**5**	**1**	**2**	**2**		
Methanoculleus	A42	1	1			1			*Methanoculleus palmaeoli*; Y16382	96
									Uncultured archaeon clone PL-9A5; AY570665	98
	A43					1			*Methanoculleus palmaeoli*; Y16382	97
									Uncultured archaeon clone PL-9A5; AY570665	99
	A47	2	8						*Methanoculleus palmaeoli*; Y16382	
Total		**3**	**9**	**0**	**0**	**2**	**0**	**0**		
Methanosarcinales	A57					1			*Methanomethylovorans hollandica* strain ZB (AY260433)	99
Methanomethylovorans	A58					1			*Methanomethylovorans hollandica* strain ZB (AY260433)	99
	A59	6	6		3	3	1	6	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
	A60		2	1					*Methanomethylovorans hollandica* strain ZB (AY260433)	99
	A61		1						*Methanomethylovorans hollandica* strain ZB (AY260433)	99
	A62		1			1			*Methanomethylovorans hollandica* strain ZB (AY260433)	99
Total		**6**	**10**	**0**	**3**	**6**	**1**	**6**		
Methanosaeta	A63			1					*Methanosaeta concilii* (X51423)	99
	A64	39	31	39	37	25	48	29	*Methanosaeta concilii* (X51423)	99
	A65	22	30	42	26	21	25	19	*Methanosaeta* sp. (AB077211)	98
	A66		1						*Methanosaeta* sp. Clone A1 (AJ133791)	99
	A67				1				*Methanosaeta* sp. Clone A1 (AJ133791)	99
	A68					1			*Methanosaeta* sp. Clone A1 (AJ133791)	99
	A69					1			*Methanosaeta* sp. Clone A1 (AJ133791)	99
	A70							1	*Methanosaeta* sp. Clone A1 (AJ133791)	99
	A71				1				*Methanosaeta* sp. Clone A1 (AJ133791)	98
	A73	4	5		2	2	5	2	*Methanosaeta* sp. Clone A1 (AJ133791)	98
	A74		1				1		*Methanosaeta* sp. Clone A1 (AJ133791)	99
Total		**65**	**68**	**82**	**67**	**50**	**79**	**51**		

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		Number of clones detected								
		MLSB				WIP				% Similarity (GenBank)
	OTU	6 m	10 m	20 m	30 m	6 m	10 m	20 m	Closest Related Species or Clone	
Unclassified	A96			1	1				Crenarchaeotal sp. clone pJP (L25301)	92
Crenarchaeota	A101				2				Uncultured archaeon clone:OHKA4.59 (AB094541)	97
	A102						1		Uncultured archaeon clone:ASC40 (AB161339)	96
	A104							1	Unidentified archaeon clone 122 (AJ831142)	99
	A108					1			Uncultured archaeon clone ss043 (AJ969795)	93
Total		**0**	**0**	**1**	**3**	**1**	**1**	**1**		
Chimeras		**12**	**2**	**6**	**14**	**9**	**7**	**20**		
Clone Library Size		**87**	**90**	**91**	**93**	**80**	**92**	**83**		

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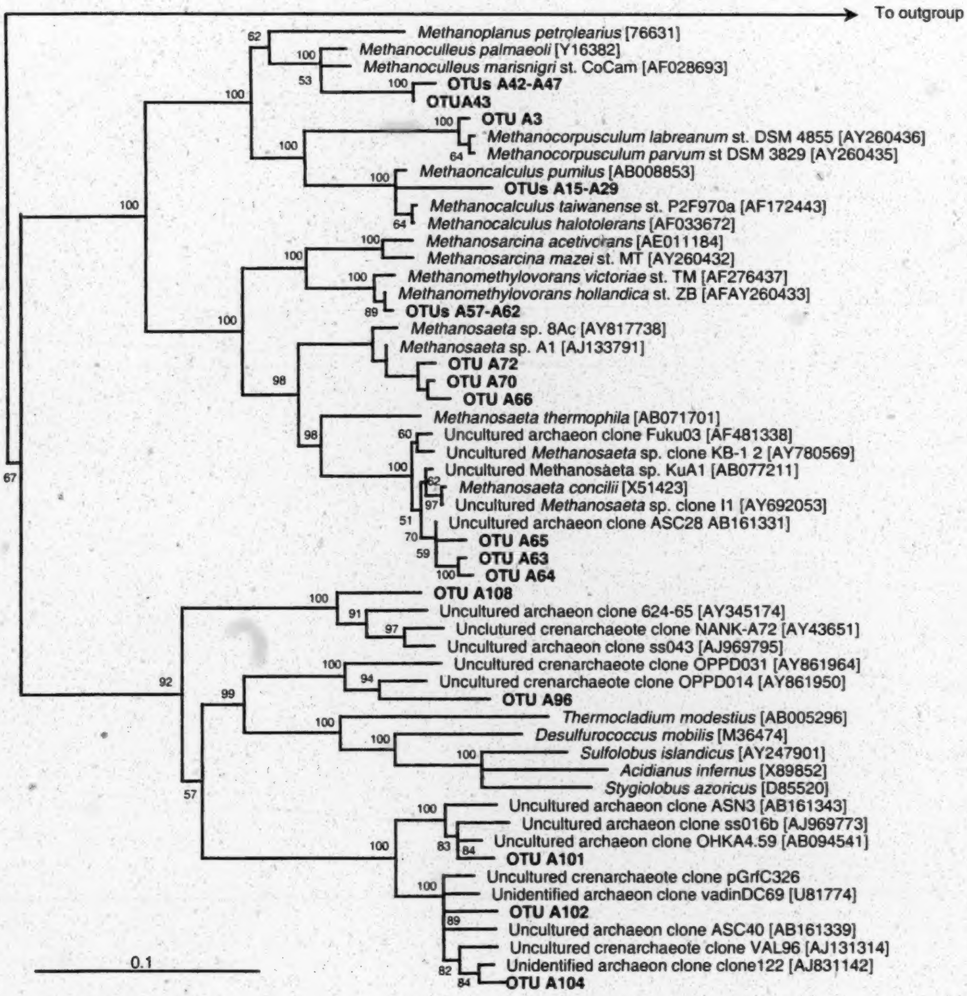
To outgroup
Methanoplanus petrolearius [76631]
Methanoculleus palmaeoli [Y16382]
Methanoculleus marisnigri st. CoCam [AF028693]
OTUs A42-A47
OTUA43
OTU A3
Methanocorpusculum labreanum st. DSM 4855 [AY260436]
Methanocorpusculum parvum st DSM 3829 [AY260435]
Methaoncalculus pumilus [AB008853]
OTUs A15-A29
Methanocalculus taiwanense st. P2F970a [AF172443]
Methanocalculus halotolerans [AF033672]
Methanosarcina acetivorans [AE011184]
Methanosarcina mazei st. MT [AY260432]
Methanomethylovorans victoriae st. TM [AF276437]
Methanomethylovorans hollandica st. ZB [AFAY260433]
OTUs A57-A62
Methanosaeta sp. 8Ac [AY817738]
Methanosaeta sp. A1 [AJ133791]
OTU A72
OTU A70
OTU A66
Methanosaeta thermophila [AB071701]
Uncultured archaeon clone Fuku03 [AF481338]
Uncultured Methanosaeta sp. clone KB-1 2 [AY780569]
Uncultured Methanosaeta sp. KuA1 [AB077211]
Methanosaeta concilii [X51423]
Uncultured Methanosaeta sp. clone I1 [AY692053]
Uncultured archaeon clone ASC28 AB161331]
OTU A65
OTU A63
OTU A64
OTU A108
Uncultured archaeon clone 624-65 [AY345174]
Unclutured crenarchaeote clone NANK-A72 [AY43651]
Uncultured archaeon clone ss043 [AJ969795]
Uncultured crenarchaeote clone OPPD031 [AY861964]
Uncultured crenarchaeote clone OPPD014 [AY861950]
OTU A96
Thermocladium modestius [AB005296]
Desulfurococcus mobilis [M36474]
Sulfolobus islandicus [AY247901]
Acidianus infernus [X89852]
Stygiolobus azoricus [D85520]
Uncultured archaeon clone ASN3 [AB161343]
Uncultured archaeon clone ss016b [AJ969773]
Uncultured archaeon clone OHKA4.59 [AB094541]
OTU A101
Uncultured crenarchaeote clone pGrfC326
Unidentified archaeon clone vadinDC69 [U81774]
OTU A102
Uncultured archaeon clone ASC40 [AB161339]
Uncultured crenarchaeote clone VAL96 [AJ131314]
Unidentified archaeon clone clone122 [AJ831142]
OTU A104
62
100
53
100
100
100
100
64
100
64
100
100
100
100
89
100
98
98
60
100
62
97
51
70
59
100
67
100
91
97
100
94
92
99
100
100
100
57
100
100
83
84
100
89
82
84
0.1

Figure 3-21: Phylogenetic tree showing relationships of Archaeal 16S rRNA gene sequences obtained from neat MFT samples to known Archaeal sequences. Clones fall into clusters indicated on the tree by one representative. The tree was constructed from partial (~1000 bp) 16S rRNA sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequences difference. The tree was rooted using *E. coli* as an out-group.

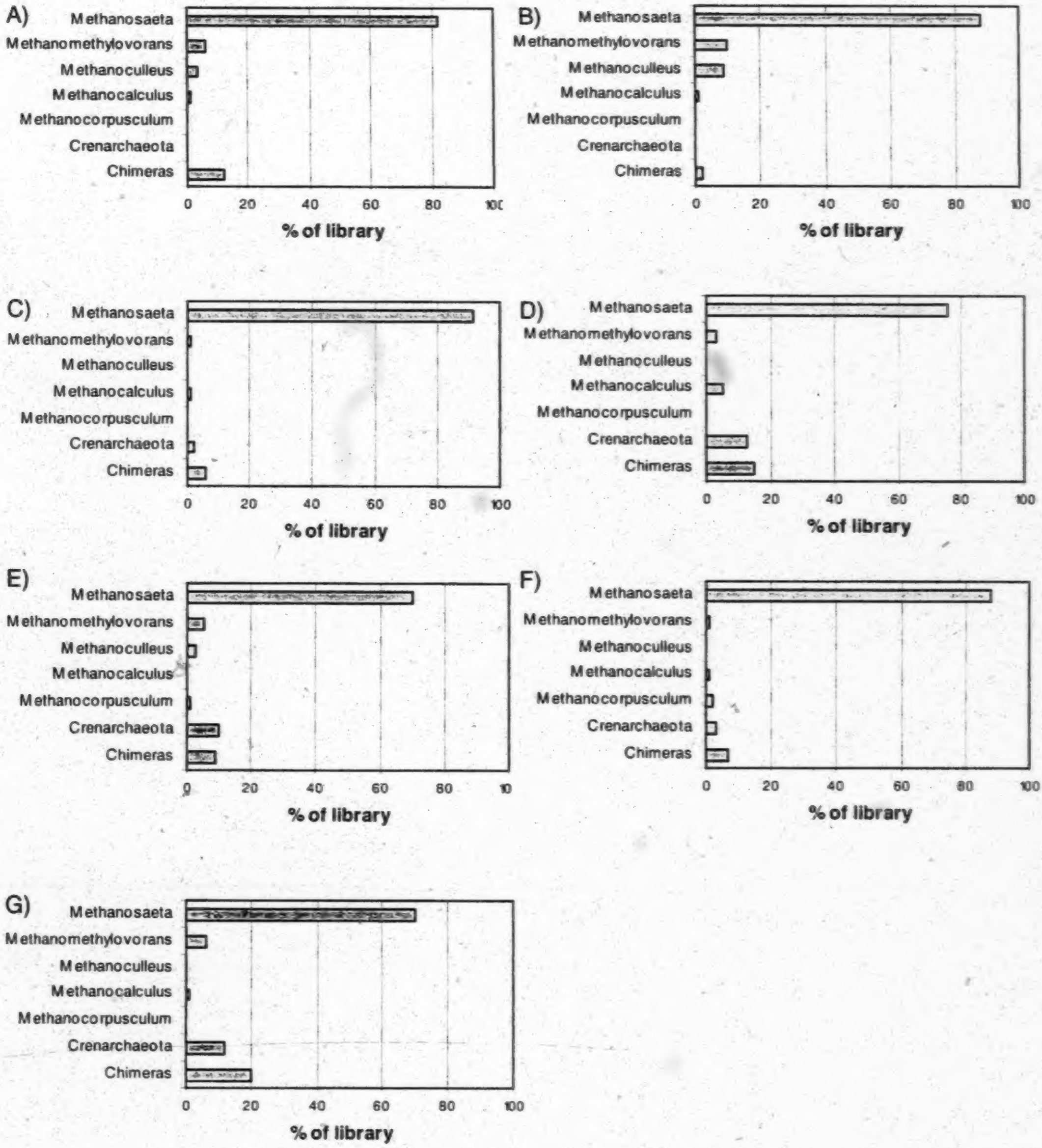

Figure 3-22: Archaeal genera and groups most closely related to sequences isolated from neat tailings samples taken from MLSB and WIP. A) MLSB 6 m; B) MLSB 10 m; C) MLSB 20 m; D) MLSB 30 m; E) WIP 6 m; F) WIP 10 m; G) WIP 20 m.

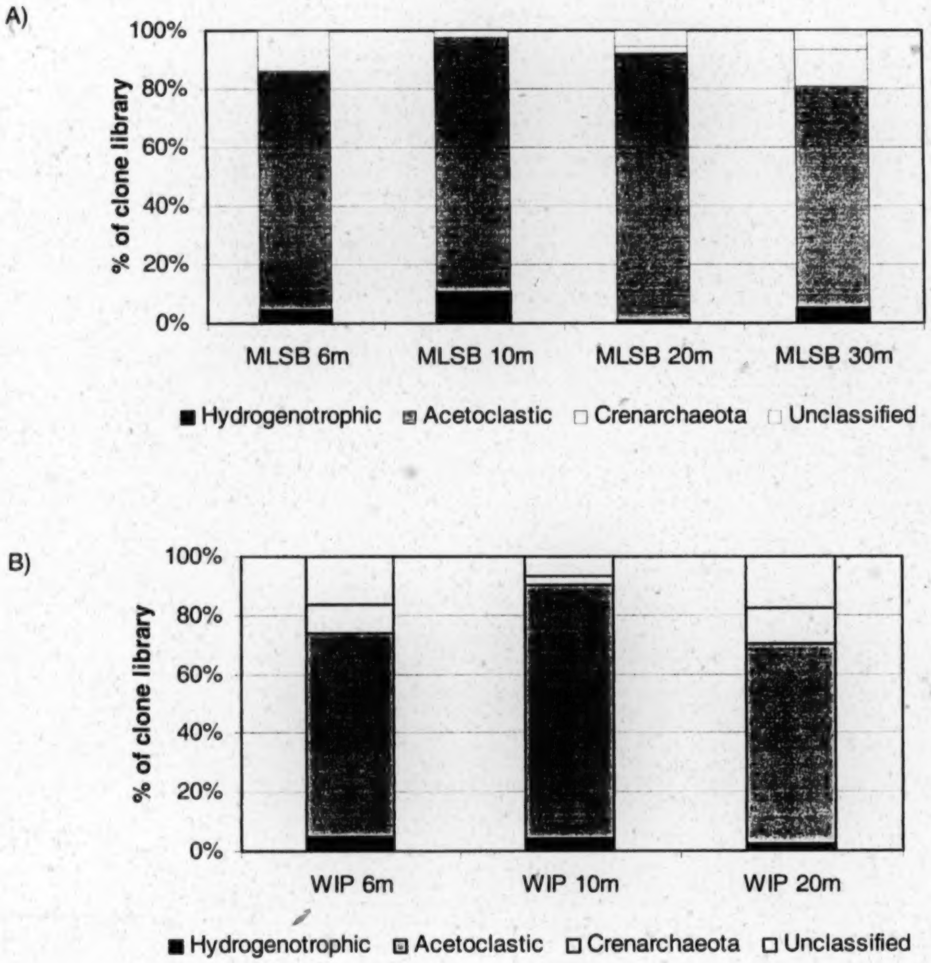

Figure 3-23: Methanogenic species in MFT with similar metabolic capabilities are grouped together. A) MFT from MLSB. B) MFT from WIP.

In addition to Archaeal clones falling into the kingdom *Euryarchaeota*, several clones related to members of the *Crenarchaeota* were detected. The kingdom *Crenarchaeota* initially contained only Archaeal species isolated from hyperthermophilic environments. However, as more environments were surveyed using 16S rRNA gene based non-culture techniques, it was discovered that a large number of microorganisms affiliated with this kingdom were obtained from mesophilic and psychrophilic environments (Madigan et al., 2000). Some non-hyperthermophilic environments from which *Crenarchaeotal* sequences have been obtained include marine water and sediment, sea ice, Antarctic sediment, and as symbionts of marine sponges (Madigan et al., 2000). None of these non-extremophilic *Crenarchaeota* has ever been cultured, so their physiology and metabolic capabilities are unknown. However, one can speculate on their metabolic roles based on the chemistry of the environments in which they were detected, and the characteristics of cultured Crenarchaeotal species. Uncultured *Crenarchaeota* have been detected in aerobic and anaerobic environments, and probably use hydrogen and sulfur as energy sources (Dawson et al., 2000).

The clones related to members of the kingdom *Crenarchaeota* fall into three distinct groups (Figure 3-21). Two groups fall into a large clade of *Crenarchaeota* named the Miscellaneous *Crenarchaeota* Group (MCG) by Inagake et al. (2003). The second group forms its own clade along with sequences from microorganisms retrieved from diverse environments, namely a hyperthermophilic deep sea vent (Page et al., 2004), the Obsidian Pool in Yellowstone National Park (Barns et al., 1994), and landfill leachate. It is very difficult to classify Crenarchaeotal environmental isolates due to the fact that there is no consensus in the literature as to the naming of particular phylogenetic groups.

Interestingly, the largest numbers of sequences related to the *Crenarchaeota* were found in the lower depths of MLSB (20 m and 30 m) and in WIP. This may be correlated with the relative age of the tailings, keeping in mind that the age of the MFT increases with depth and that WIP contains old MFT which was pumped from MLSB. However, because we do not know the metabolic capabilities of the *Crenarchaeota* clones, I cannot speculate on their role in the tailings.

There were a large number of chimeric sequences detected in the Archaeal 16S rRNA gene clone libraries constructed from the neat tailings. The reason for this is not known, however since the RDP II database is used to detect the chimeras, and since Archaeal sequences are not well represented in this database, chimeras may have been incorrectly assigned. The suspected chimeric sequences were very distantly related to isolated Euryarchaeotal and Crenarchaeotal species, so to be on the safe side these clones were not included in further analyses.

3.3.5 Bacterial Species in the tailings

Due to the large Bacterial diversity present in the tailings, only clones from OTUs appearing two or more times were sequenced, and only the first 500 bp of the 16S rRNA gene was sequenced for the majority of the clones. Certain OTUs were selected for full-length sequencing to confirm close relationships to known species of bacteria. These sequences are indicated in Appendix 6. The numbers of each OTU found in each of the libraries, and their closest related species or clone is shown in Table 3-9.

The Bacterial clone libraries were largely made up of Proteobacteria, making up anywhere from 30 to 55% of each library (Figure 3-24, Figure 3-25). The Proteobacteria are a large, metabolically diverse group of Gram negative bacteria. Phylogenetically, the Proteobacteria are all related, although they display a wide range of morphological, metabolic and physiologic traits. The Proteobacteria are split into five subgroups, α-, β-, γ-, δ-, and ε-Proteobacteria (Figure 3-26). A smaller number of clones were related to other groups of bacteria, including Chloroflexi, Spirochaetes, Clostridia and Acholeplasma (Figure 3-24, Figure 3-25). These groups are discussed in more detail below.

Several OTUs detected in the neat MFT samples were found to be sequences very closely related to a laboratory strain of *Escherichia coli*, and were subsequently eliminated from further analysis of the clone libraries. The presence of *E. coli* itself may not be that unusual, because it may have been introduced through raw sewage received by MLSB in the past (M. MacKinnon, personal communication), however due to the high similarity of these clones to *E. coli* strain K12, it was assumed to be a result of laboratory contamination. *E. coli* strain K12 is the host bacterium used to make the clone libraries. Because *E. coli* sequence contamination appeared in all of the libraries, and because all negative controls indicated that contamination during the DNA extraction step and PCR amplification did not occur, the MFT samples themselves may contain *E. coli*. If this is a result of contamination, this may have happened before the MFT samples were received in the laboratory, since they may not have been handled aseptically, or it could have occurred during the manipulations of the tailings, even though aseptic technique was used. The OTUs representing *E. coli* sequences were excluded from further study because it is assumed that they are not native members of the microbial communities in the MFT. The presence of *E. coli* contamination decreases the value of these clone libraries, because E. coli sequences made up 10 to 20% of the clones. However, I can still use these libraries to obtain an initial picture of Bacterial diversity in the *in situ* MFT, and conduct future sampling and experiments more carefully.

Table 3-9: Bacterial OTUs found in neat MFT from MLSB and WIP. Number of clones in each OTU, percent similarity to closest related species or clone, and total number of clones in each taxonomic group are shown.

	MLSB					WIP			% similarity	
OTU	6m	10m	20m	30m	60m	10m	20m	Closest related species or clone [a]	GenBank	RDP II
Alphaproteobacteria										
B1		1		2				*Caulobacter* sp.; DSM 10556; AJ227788	98	0.964
B2								magnetite-containing magnetic vibrio; MV1; L06455	94	0.711
					2			*Hyphomicrobium zavarzinii* (T); ZV-622; Y14305	–	0.589
B3					2			*Sphingomonadaceae* bacterium MWH-CaK2; AJ565420	94	0.787
B4								uncultured bacterium; B3NR56D5; AY957908	98	0.987
		1				1		Uncultured *Azospirillum* sp. clone GCPF16; AY129790	97	–
Total	**0**	**2**	**0**	**2**	**4**	**1**	**0**			
Betaproteobacteria										
B5	5	2			2	3	1	beta proteobacterium PB7; AY686732	98	0.971
B6								uncultured beta proteobacterium; B-Y34; AY622248	98	0.903
	6	4			3	4		*Rhodoferax ferrireducens* (T); T118; AF435948	97	0.882
B7								uncultured beta proteobacterium; B-Y34; AY622248	97	0.889
		1	2	1	4	2	1	*Rhodoferax ferrireducens* (T); T118; AF435948	97	0.880
B8								uncultured beta proteobacterium; B-Y34; AY622248	97	0.896
	1					2	1	*Rhodoferax ferrireducens* (T); T118; AF435948	97	0.887
B9								uncultured beta proteobacterium; B-Y34; AY622248	97	0.899
					1	1		*Rhodoferax ferrireducens* (T); T118; AF435948	96	0.880
B10								uncultured bacterium; RB13C10; AF407413	97	0.947
						3		*Rhodoferax antarcticus*; Fryx1; AY609198	96	0.925
B11								uncultured bacterium; RB9C1; AF407392	98	0.973
					2			*Rhodoferax antarcticus*; Fryx1; AY609198	97	0.933
B12		2	2	1	1	3	1	*Acidovorax defluvii* (T); BSB411; Y18616	99	0.963
B13								*Acidovorax defluvii* (T); BSB411; Y18616	98	0.924
			5					denitrifying Fe<II>-oxidizing bacteria; BrG1; U51101	99	0.937
B14		1			1	2		*Acidovorax facilis*; DSM 649; AJ420324	98	0.902
B15								*Malikia granulosa*; type strain: P1; AJ627188	98	0.900
		2					1	*Hydrogenophaga taeniospiralis* (T); ATCC 49743; AF078768	98	0.873
B16			2					*Hydrogenophaga taeniospiralis* (T); ATCC 49743; AF078768	99	0.947
B17			3					uncultured beta proteobacterium; AKYG1037; AY921769	97	0.882
B18								beta proteobacterium CDB21; AB194096	95	0.850
	1	1						*Sterolibacterium denitrificans* (T); Chol-1S; AJ306683	–	0.729
B19								uncultured beta proteobacterium; ccs265; AY133064	96	0.814
		1					1	*Propionivibrio limicola* (T); GolChi1 T; AJ307983	–	0.705
B20								uncultured beta proteobacterium; ccs265; AY133064	95	0.812
	5	6	4					*Thiobacillus aquaesulis*; U58019	–	0.738
B21								Uncultured soil bacterium clone L1A.6E11; AY988982	98	–
	7	22	5	5	2	1	6	*Thiobacillus denitrificans* (T); NCIMB 9548; AJ243144	95	0.790
B22								beta proteobacterium 5Z-C1; AJ224618	98	0.928
	1	2						*Thiobacillus denitrificans* (T); NCIMB 9548; AJ243144	96	–

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OTU	MLSB					WIP		Closest related species or clone [a]	% similarity	
	6m	10m	20m	30m	60m	10m	20m		GenBank	RDP II
B23								uncultured bacterium; RB7C6; AF407385	99	0.949
	1		2					*Thiobacillus denitrificans* (T); NCIMB 9548; AJ243144	94	0.894
B24								uncultured bacterium; 35-16K; AY955090	96	0.870
			2					*Thiobacillus thioparus*; AF005628	94	–
B26								uncultured bacterium; RB7C6; AF407385	99	1.000
				1		1		uncultured *Thiobacillus* sp. KF-Gitt2-40; AJ295643	98	0.930
B27								uncultured bacterium; RB7C6; AF407385	99	0.972
			1				1	uncultured *Thiobacillus* sp. KF-Gitt2-40; AJ295643	97	0.880
B28		1			4			uncultured bacterium; RB7C6; AF407385	99	0.950
B29								uncultured beta proteobacterium; ccspost271; AY133108	98	0.948
			1	3				*Thiobacillus denitrificans* strain NCIMB; AJ243144	95	–
B30	1				1			uncultured beta proteobacterium; 36-9; AF351219	96	0.925
B31					1		1	uncultured beta proteobacterium; 36-9; AF351219	97	0.862
B32			1	8				uncultured beta proteobacterium; 36-9; AF351219	98	0.886
B33								beta proteobacterium Rufe9; AY235687	92	–
		2						*Denitratisoma oestradiolicum*; AcBE2-1; AY879297	–	0.741
B34								beta proteobacterium MWH-UniP1; AJ565421	97	0.897
		3						Burkholderia cepacia (T); ATCC 25416T; U96927	–	0.711
B35								uncultured bacterium; UTFS-OF08-18; AB166784	94	0.816
			1				2	*Dechloromonas* sp. NM; AF170355	94	0.800
B36								uncultured bacterium; 35-45D; AY955092	97	0.904
			1		2			*Thauera phenylacetica* (T); B4P = DSM 14743ᵀ; AJ315678	97	0.876
B37								*Azoarcus* sp.; M3; Y11041	–	0.696
						2		uncultured *Methylophilaceae* bacterium; 10-3Ba28; AY360550	–	0.700
Total	**28**	**50**	**32**	**19**	**24**	**24**	**16**			
Gammaproteobacteria										
B38								uncultured bacterium; 5H_90; AY546512	97	0.892
		2						*Dokdonella koreensis*; DS-123; AY987368	–	0.737
B39								uncultured bacterium; 5H_90; AY546512	–	0.908
		3						*Lysobacter gummosus*; KCTC 12132; AB161361	–	0.722
B40								uncultured bacterium; 5H_90; AY546512	97	0.906
		4						*Xanthomonas campestris*; XCC15; AF123092	–	0.716
B41								Uncultured Gammaproteobacteria clone 4P36; AJ871058	99	–
			2					*Dokdonella koreensis*; DS-123; AY987368	92	0.733
B42								Uncultured eubacterium clone IAFR510; AF270959	96	–
	2	3	5		1	4		*Thialkalivibrio thiocyanodenitrificans* strain ARhD; AY360060	88	–
B43								Uncultured eubacterium clone IAFR510; AF270959	95	–
		4				1		*Methylocaldum* sp. E10a; AJ868426	90	0.586
B44								Uncultured eubacterium clone IAFR510; AF270959	95	–
			2				2	Codakia costata gill symbiont; L25712	–	0.607
B45								endosymbiont of *Seepiophila jonesi*; AY129105	–	0.598
	1				2			Uncultured soil bacterium clone PYR10d11; DQ123671	95	–

Continued on next page

OTU	MLSB					WIP		Closest related species or clone ᵃ	% similarity	
	6m	10m	20m	30m	60m	10m	20m		GenBank	RDP II
B46								Uncultured eubacterium clone IAFR510; AF270959	97	–
		5						*Thialkalivibrio thiocyanodenitrificans* strain ARhD; AY360060	89	–
B47			1		1			Uncultured eubacterium clone IAFR510; AF270959	100	–
B48								Uncultured bacterium clone CCSD_DF1080_B22; AY820699	94	–
			3					*Methylocaldum* sp. E10a; AJ868426	–	–
B49								Uncultured eubacterium clone IAFR510; AF270959	98	–
			1			1		*Halomonas* sp. A-9; AY914060	–	0.608
B50								Codakia costata gill symbiont; L25712	–	0.620
		1			1		1	Thialkalivibrio thiocyanodenitrificans strain ARhD; AY360060	90	–
B51								Uncultured gamma proteobacterium clone LTUG09056; AY144261	91	–
		3		1				*Methylocaldum szegediense* (T); OR2; U89300	96	–
B52								uncultured gamma proteobacterium; B-AC40; AY622251	99	0.958
			2					*Methylosarcina fibrata* (T); AML-C10; AF177296	96	0.697
B53								uncultured gamma proteobacterium; B-AC40; AY622251	98	0.939
			4	2	3			phototrophic bacterium (T); DSM 2111; X93478	93	0.674
B54								uncultured gamma proteobacterium; B-AC40; AY622251	95	0.946
				2				phototrophic bacterium (T); DSM 2111; X93478	–	0.710
B55								uncultured bacterium; TSBX24; AB186843	93	0.725
					2			Microbulbifer salipaludis (T); SM-1; AF479688	–	0.657
B56								uncultured bacterium; 69-7G; AY955095	98	0.910
		1			1			*Pseudomonas alcaligenes*; LB19; AF390747	97	0.844
B57						1	1	Pseudomonas sp. 10BSH1; DQ011926	99	1.000
Total	3	26	20	5	11	7	4			
Deltaproteobacteria										
B58								uncultured bacterium; KNA6-EB15; AB179691	94	0.845
		2				1		*Desulfobulbus* sp. BG25; U85473	97	–
B59								uncultured bacterium; KNA6-EB15; AB179691	95	0.811
				1			1	*Syntrophomonas* wolinii 16S rRNA gene; X7090	87	–
B60								uncultured bacterium; 1013-1-CG26; AY532547	97	0.885
						2		*Methylocaldum* sp. E10a; AJ868426	88	–
B61						1	1	uncultured bacterium; KNA6-EB15; AB179691	94	0.761
B62							2	uncultured proteobacterium; ccs202; AY133065	97	0.904
B63								uncultured bacterium; KNA6-EB15; AB179691	95	0.768
					2			Desulfobulbus sp. BG25; U85473	89	–
B64								uncultured delta proteobacterium; ML320J-28; AF458285	89	0.700
	1			1		2		*Desulfocapsa thiozymogenes* (T); DSM 7269; X95181	–	0.512
B65								uncultured bacterium; sipK108; AJ307944	95	0.815
					1	1	2	*Desulfocapsa* sp. Cad626; AJ511275	96	0.796
B66	1				1			uncultured bacterium; SHA-42; AJ306771	92	0.693
B67					2			uncultured delta proteobacterium 36-11; AF351220	97	0.833
B68								uncultured bacterium; FW99; AF523966	93	0.732
					2			uncultured *Syntrophus* sp.; PD-UASB-35; AY261813	92	0.679

Continued on next page

	MLSB					WIP			% similarity	
OTU	6m	10m	20m	30m	60m	10m	20m	Closest related species or clone *	GenBank	RDP II
B69								uncultured bacterium; FW99; AF523966	94	0.726
					2			uncultured *Syntrophus* sp.; PD-UASB-40; AY261815	91	0.656
B70								uncultured eubacterium WCHB1-12; AF050534	99	0.974
					2			*Syntrophus* sp. 16S rRNA gene, partial, Clone B3; AJ133796	94	0.786
B72								uncultured delta proteobacterium; 44a-B1-10; AY082457	92	0.685
		2						uncultured *Desulfosarcina* sp.; SB4_53; AY177791	–	0.665
B73						1	1	uncultured delta proteobacterium; HMMVPog-2; AJ704678	93	0.737
B74								uncultured bacterium; KM88; AY216443	–	0.647
	1					1		*Desulfobacterium indolicum*; AJ237607	89	–
B75								uncultured bacterium; KM88; AY216443	–	0.645
							2	*Desulfobacterium cetonicum* (T); DSM 7267; AJ237603	95	0.603
B76					2			Unidentified bacterium clone Qui4P2-36; AJ518578	97	–
Total	**3**	**4**	**0**	**2**	**14**	**9**	**9**			
CFB										
B77						1	1	uncultured bacterium; Gitt-KF-184; AJ532698	94	0.765
Total	**0**	**0**	**0**	**0**	**0**	**1**	**1**			
Chloroflexi										
B81			2					uncultured bacterium; 2-3; AY548942	95	0.824
B82	2							uncultured bacterium; FA2; AY553935	98	0.933
Total	**2**	**0**	**2**	**0**	**0**	**0**	**0**			
Spirochaetes										
B84								uncultured bacterium; E5; AY426470	96	0.903
		1	1					*Spirochaetes* bacterium SA-8; AY695839	92	0.738
Total	**0**	**1**	**1**	**0**	**0**	**0**	**0**			
Clostridia										
B100								uncultured bacterium; Eub No. 20; AF395430		0.876
		1	3					*Clostridium aminobutyricum*; DSM 2634; X76161	96	0.843
B101				4				*Clostridium aminobutyricum*; DSM 2634; X76161	95	0.833
B111								Desulfotomaculum kuznetsovii; 17; AY036904	91	0.530
		18	2			2		toluene-deg.methanogenic consort.bact Eub 1; AF423181	94	0.555
B112								uncultured bacterium; GIF17; AF407206	94	0.810
		2						*Desulfotomaculum kuznetsovii*; 17; AY036904	92	0.594
B113								*Desulfotomaculum thermobenzoicum*; DSM 6193; 11; AJ294430	92	0.507
			3					uncultured low G+C Gram-positive bacterium; 36-20; AF351221	94	0.551
B114								uncultured low G+C Gram-positive bacterium; 36-20; AF351221		0.555
	2							*Desulfotomaculum gibsoniae*; DSM 7213; 19A; AJ294431	91	0.528
B115								*Sporotomaculum syntrophicum* (T); FB; AB076610	–	0.538
						5		toluene-degrading meth consort bact; Eub 1; AF423181	94	0.597
B119	1	1						*Moorella thermoacetica*; "PT1 = DSM 12993"; AJ633105	–	0.420
Total	**4**	**21**	**8**	**16**	**0**	**7**	**0**			

Continued on next page

	MLSB					WIP			% similarity	
OTU	6m	10m	20m	30m	60m	10m	20m	Closest related species or clone [a]	GenBank	RDP II
Acholeplasma										
B120								uncultured bacterium; EUB33-2; AY693836	96	0.829
	1				1			*Acholeplasma palmae* (T); ATCC49389; J233; L33734	90	0.627
B121								uncultured bacterium; TANB25; AY667255	96	0.796
	2							*Acholeplasma palmae* (T); ATCC49389; J233; L33734	91	0.642
B122								uncultured bacterium; EUB33-2; AY693836	95	0.862
		1		1				*Acholeplasma parvum*; H23M; AY538170	–	0.660
B125	1					1		*Acholeplasma parvum*; H23M; AY538170	87	0.517
B126								*Acholeplasma vituli* (T); FC-097; AF031479	–	0.459
								Clostridium cocleatum (T); DSM 1551; Y18188	–	0.434
								Bacillus solfatarensis; Sol1; AY518549	–	0.430
	1						1	*Allofustis seminis* (T); CCUG 45438; AJ410303	–	0.440
B127								uncultured bacterium; EUB33-2; AY693836	96	0.817
					1	1		*Acholeplasma brassicae*; 0502; AY538163	92	0.621
Total	**5**	**1**	**0**	**1**	**2**	**2**	**1**			
E.coli										
B128	11	17	2	12	6	15	14	E. coli K12		
B129	2	10		12		2		E. coli K12		
B130						2		E. coli pk3	97	0.985
B131							2	E. coli pK3		
B132						2		E. coli pK3		
Total	**13**	**27**	**2**	**24**	**6**	**19**	**14**			
Unsequenced										
Total	**35**	**35**	**21**	**34**	**27**	**22**	**40**			
Total Clones	**94**	**178**	**86**	**103**	**88**	**96**	**87**			

[a] when closest match was an uncultured clone, the closest cultured species is also shown when % similarity is >85%

[b] Ribosomal Database Project II, http://rdp.cme.msu.edu/index.jsp; value is similarity index (S_{ab}), representing the similarity between the two sequences

[c] – indicates that this sequence was not listed as a close match (>80% for GenBank, >0.500 for RDP-II)

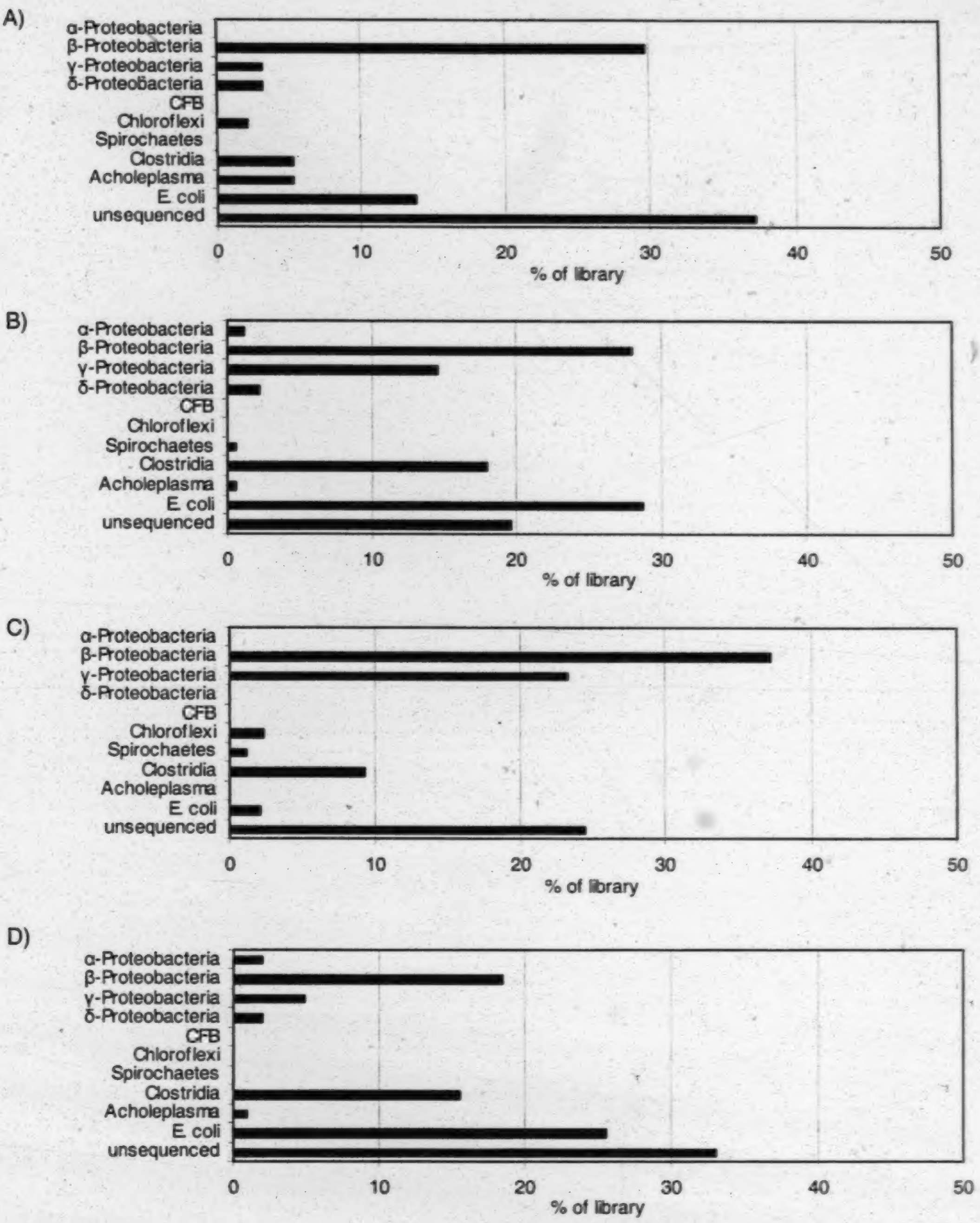

Figure 3-24: Distribution of Bacterial clones with depth in libraries constructed from MLSB. A) MLSB 6 m; B) MLSB 10 m; C) MLSB 20 m; D) MLSB 30 m.

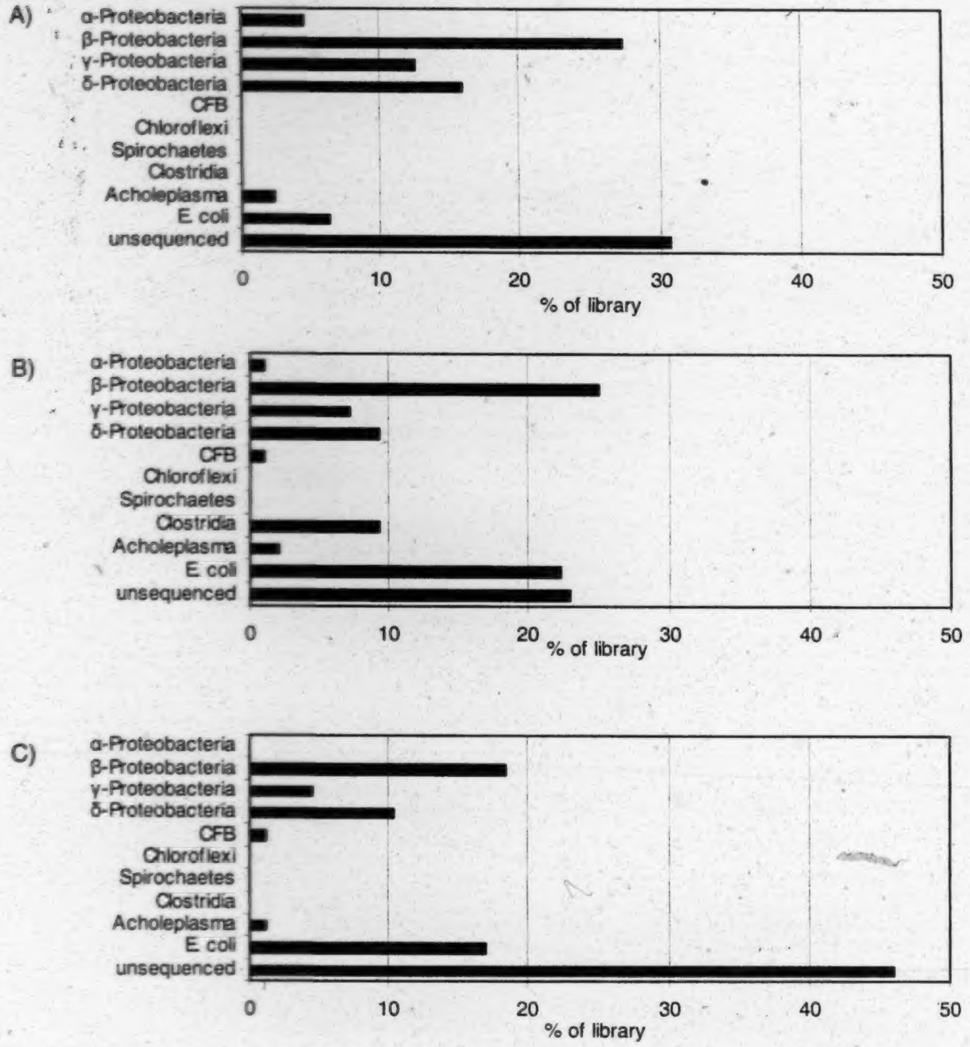

Figure 3-25: Distribution of Bacterial clones with depth in libraries constructed from WIP. A) WIP 6 m; B) WIP 10 m; C) WIP 20 m.

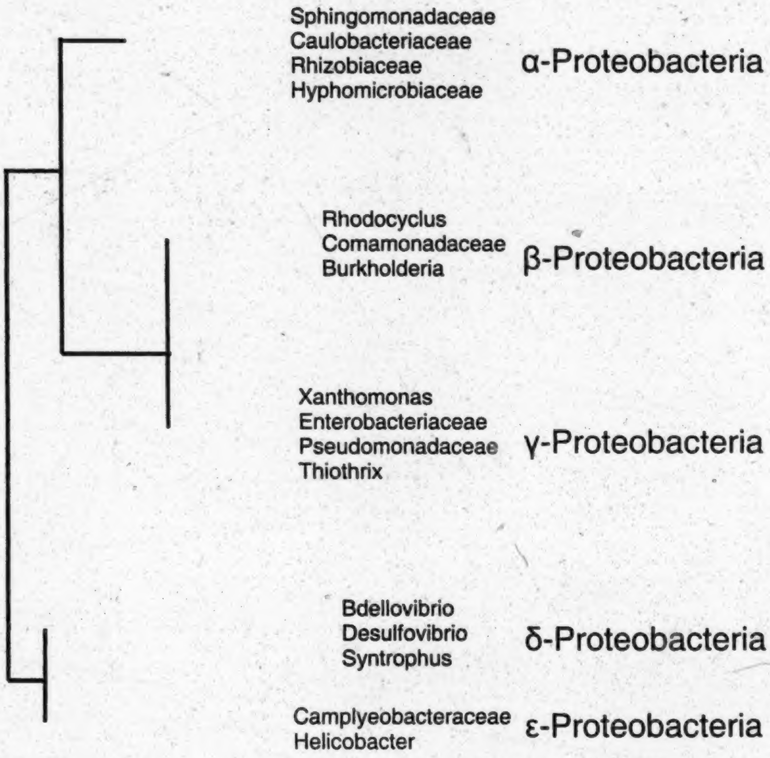
Sphingomonadaceae
Caulobacteriaceae
Rhizobiaceae
Hyphomicrobiaceae
α-Proteobacteria
Rhodocyclus
Comamonadaceae
Burkholderia
β-Proteobacteria
Xanthomonas
Enterobacteriaceae
Pseudomonadaceae
Thiothrix
γ-Proteobacteria
Bdellovibrio
Desulfovibrio
Syntrophus
δ-Proteobacteria
Camplyeobacteraceae
Helicobacter
ε-Proteobacteria

Figure 3-26: Phylogenetic tree of the proteobacteria. Adapted from Madigan et al. (2000).

3.3.6 α-Proteobacteria

α-Proteobacteria clones were found only in very low numbers in the libraries constructed from MLSB 10 m, MLSB 30 m, WIP 6 m and WIP 10 m samples (Figure 3-25). The α-proteobacteria includes many oligotrophic species, which can grow at very low nutrient concentrations.

OTU B1 is related to the genus *Caulobacter* (Figure 3-27), which are unusual in that they produce prostheca which are used to attach themselves to surfaces (Prescott et al., 1999). This unusual physical characteristic may allow *Caulobacter* spp. to increase their nutrient uptake by providing an increased surface area. *Caulobacter* are ubiquitous in aerobic zones of aqueous environments (Poindexter, 1999), which makes it unusual to find them in the extremely anaerobic zones of the tailings. *Caulobacter* spp. may be present in the aerobic zones of the water above the tailings, and their DNA was transferred to the tailings in the form of detritus or through contamination during sampling, and were subsequently detected in the extracted DNA.

OTU B2 is related to a magnetite-containing vibrio clone. These unique motile bacteria have iron-containing magnetic particles called magnetosomes that allow the bacteria to sense the earth's geomagnetic field and orient themselves as they swim (DeLong et al., 1993). These are interesting bacteria, however their presence in the tailings is not clear.

OTU B3 is related to the genus *Sphingomonas* (Figure 3-27), which is made up of aerobic bacteria that are able to degrade a wide range of organic compounds including aromatic molecules, and can also hydrolyze polysaccharides into monomers (Balkwill et al., 2001). These microorganisms may be involved in the initial oxidative attack of hydrocarbon molecules in the upper aerobic zones of the tailings. Most species of *Sphingomonas* are also capable of nitrate reduction (Takeuchi et al., 2001) which may allow them to grow anaerobically in the tailings.

OTU B4 is closely related to several uncultured clones (Figure 3-27), all of which were found in drinking water or marine environments. This may indicate that these bacteria are generally found in aquatic environments.

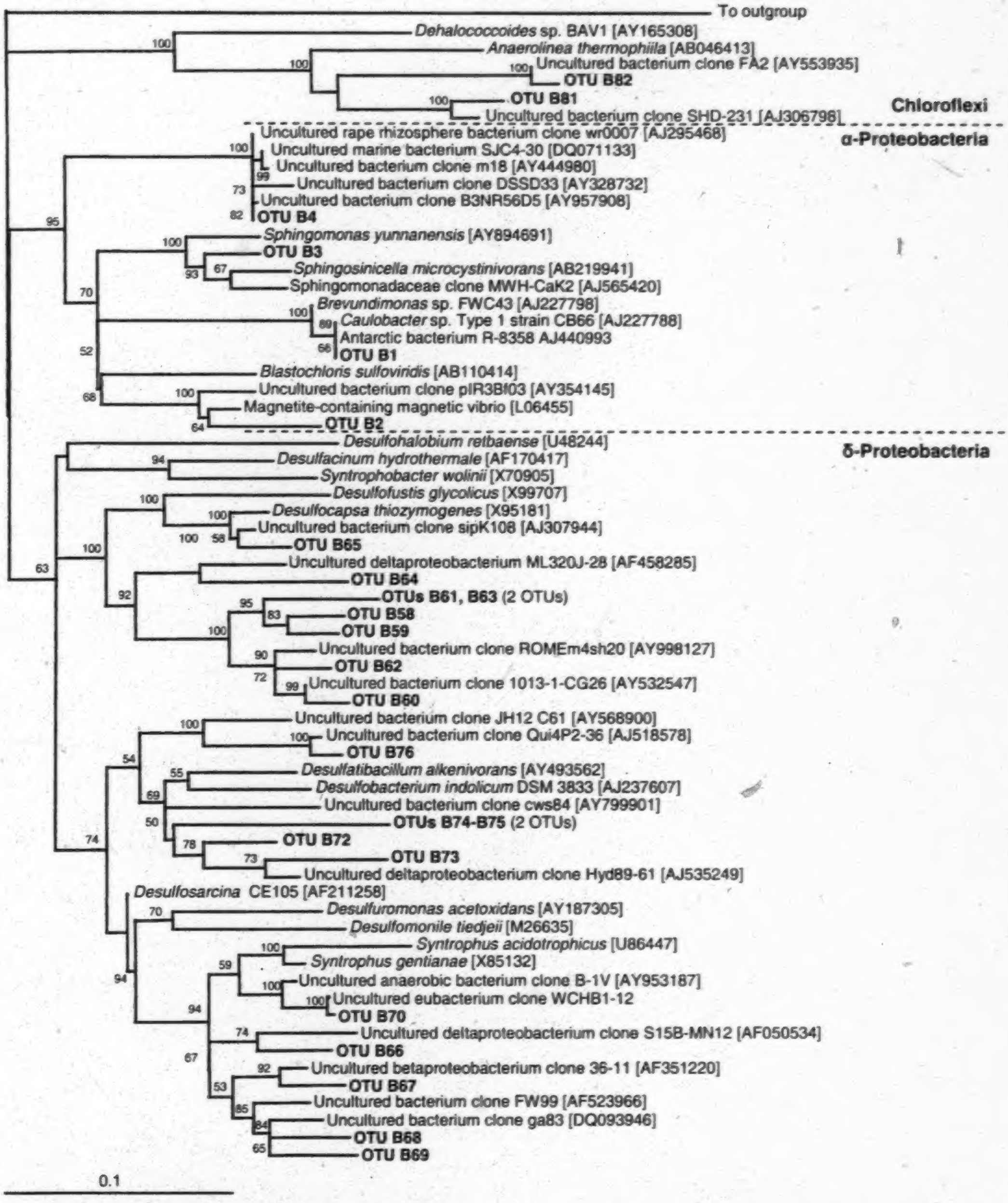
To outgroup
Dehalococcoides sp. BAV1 [AY165308]
Anaerolinea thermophiila [AB046413]
Uncultured bacterium clone FA2 [AY553935]
OTU B82
OTU B81
Uncultured bacterium clone SHD-231 [AJ306798]
Chloroflexi
Uncultured rape rhizosphere bacterium clone wr0007 [AJ295468]
α-Proteobacteria
Uncultured marine bacterium SJC4-30 [DQ071133]
Uncultured bacterium clone m18 [AY444980]
Uncultured bacterium clone DSSD33 [AY328732]
Uncultured bacterium clone B3NR56D5 [AY957908]
OTU B4
Sphingomonas yunnanensis [AY894691]
OTU B3
Sphingosinicella microcystinivorans [AB219941]
Sphingomonadaceae clone MWH-CaK2 [AJ565420]
Brevundimonas sp. FWC43 [AJ227798]
Caulobacter sp. Type 1 strain CB66 [AJ227788]
Antarctic bacterium R-8358 AJ440993
OTU B1
Blastochloris sulfoviridis [AB110414]
Uncultured bacterium clone pIR3Bf03 [AY354145]
Magnetite-containing magnetic vibrio [L06455]
OTU B2
Desulfohalobium retbaense [U48244]
δ-Proteobacteria
Desulfacinum hydrothermale [AF170417]
Syntrophobacter wolinii [X70905]
Desulfofustis glycolicus [X99707]
Desulfocapsa thiozymogenes [X95181]
Uncultured bacterium clone sipK108 [AJ307944]
OTU B65
Uncultured deltaproteobacterium ML320J-28 [AF458285]
OTU B64
OTUs B61, B63 (2 OTUs)
OTU B58
OTU B59
Uncultured bacterium clone ROMEm4sh20 [AY998127]
OTU B62
Uncultured bacterium clone 1013-1-CG26 [AY532547]
OTU B60
Uncultured bacterium clone JH12 C61 [AY568900]
Uncultured bacterium clone Qui4P2-36 [AJ518578]
OTU B76
Desulfatibacillum alkenivorans [AY493562]
Desulfobacterium indolicum DSM 3833 [AJ237607]
Uncultured bacterium clone cws84 [AY799901]
OTUs B74-B75 (2 OTUs)
OTU B72
OTU B73
Uncultured deltaproteobacterium clone Hyd89-61 [AJ535249]
Desulfosarcina CE105 [AF211258]
Desulfuromonas acetoxidans [AY187305]
Desulfomonile tiedjeii [M26635]
Syntrophus acidotrophicus [U86447]
Syntrophus gentianae [X85132]
Uncultured anaerobic bacterium clone B-1V [AY953187]
Uncultured eubacterium clone WCHB1-12
OTU B70
Uncultured deltaproteobacterium clone S15B-MN12 [AF050534]
OTU B66
Uncultured betaproteobacterium clone 36-11 [AF351220]
OTU B67
Uncultured bacterium clone FW99 [AF523966]
Uncultured bacterium clone ga83 [DQ093946]
OTU B68
OTU B69
0.1

Figure 3-27: Phylogenetic tree showing relationships of Bacterial 16S rRNA sequences obtained from neat MFT samples to known Chloroflexi, α- and δ-proteobacterial sequences. Clones fall into clusters indicated on the tree by one representative. The tree was constructed from partial (~500 bp) 16S rRNA gene sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequences difference. The tree was rooted using *M. mazei* as an out-group.

3.3.7 β-Proteobacteria

Most of the clones in the libraries constructed from the neat MFT were related to the β-proteobacteria. This division comprises a large, metabolically diverse group of bacteria, consisting of chemoheterotrophs, photolithotrophs, methylotrophs and chemolithotrophs (Prescott et al., 1999). The β-proteobacteria were found in the highest proportions in the clone libraries constructed from MLSB samples, and lower proportions from the libraries constructed from WIP samples (Figure 3-25).

One group, consisting of seven OTUs (B5 to B11), clustered tightly with an uncultured bacterium clone B-Y34 (AY622248) from a uranium-contaminated aquifer (Reardon et al., 2004), and clone L1A.11D06 (AY989355; unpublished) from soil (Figure 3-28). This cluster of clones isolated from the tailings was also approximately 97% similar to *Rhodoferax ferrireducens*, which is able to oxidize acetate with the reduction of Fe(III) (Finneran et al., 2003). The concentration of Fe (III) in the tailings ponds is not known, but the total iron concentration in MLSB ranges from 0.61 to 0.21 mg/L (Table 3-7). *R. ferrireducens* can also use nitrate as an electron acceptor (Finneran et al., 2003), and this metabolic versatility may allow it to grow on whatever electron acceptor is available in the tailings.

Three OTUs (B12 to B14), were very closely related (98 to 99%) to *Acidovorax facilis* DSM 649 (AJ420324), and to an uncultured β-proteobacterial clone FTLM27 (AF529122; unpublished) from a trichloroethene-contaminated site undergoing bioremediation (Figure 3-28). *Acidovorax facilis* is able to grow chemolithotrophically by oxidizing H_2, and so may be competing with homoacetogens and hydrogenotrophic methanogens for H_2 in the tailings (Kersters et al., 2003).

Two clones were related to an uncultured bacterium BH98-203 (AY928226; unpublished) from an aquifer (Figure 3-28). They were also closely related (98 to 99%) to *Hydrogenophaga taeniospiralis* (AF078768). These microorganisms are also able to grow autotrophically using H_2 as a source of energy (Kersters et al., 2003).

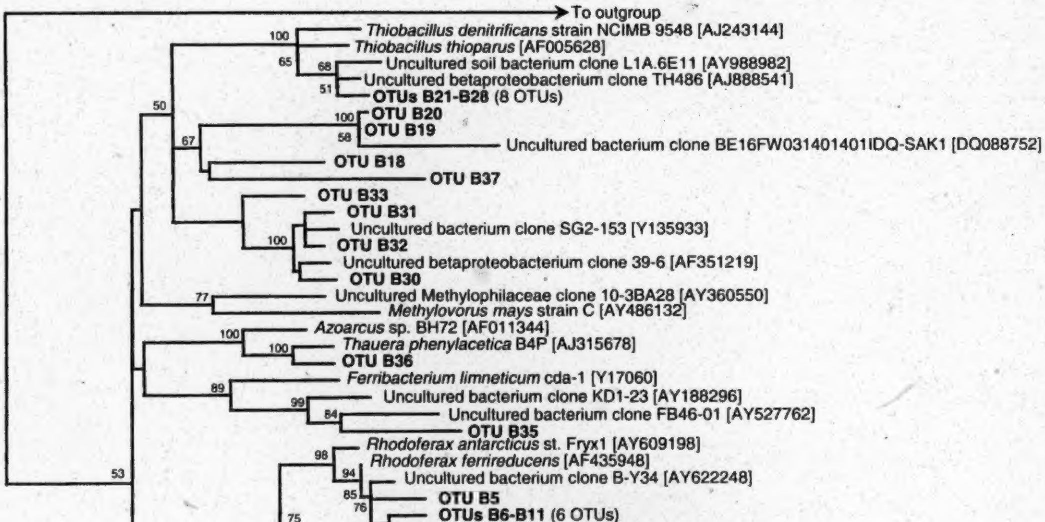
To outgroup
Thiobacillus denitrificans strain NCIMB 9548 [AJ243144]
Thiobacillus thioparus [AF005628]
Uncultured soil bacterium clone L1A.6E11 [AY988982]
Uncultured betaproteobacterium clone TH486 [AJ888541]
OTUs B21-B28 (8 OTUs)
OTU B20
OTU B19
Uncultured bacterium clone BE16FW031401401IDQ-SAK1 [DQ088752]
OTU B18
OTU B37
OTU B33
OTU B31
Uncultured bacterium clone SG2-153 [Y135933]
OTU B32
Uncultured betaproteobacterium clone 39-6 [AF351219]
OTU B30
Uncultured Methylophilaceae clone 10-3BA28 [AY360550]
Methylovorus mays strain C [AY486132]
Azoarcus sp. BH72 [AF011344]
Thauera phenylacetica B4P [AJ315678]
OTU B36
Ferribacterium limneticum cda-1 [Y17060]
Uncultured bacterium clone KD1-23 [AY188296]
Uncultured bacterium clone FB46-01 [AY527762]
OTU B35
Rhodoferax antarcticus st. Fryx1 [AY609198]
Rhodoferax ferrireducens [AF435948]
Uncultured bacterium clone B-Y34 [AY622248]
OTU B5
OTUs B6-B11 (6 OTUs)
Uncultured soil bacterium L1A.11D06 [AY989355]
Acidovorax facilis DSM 649 [AJ420324]
OTUs B12-B14 (3 OTUs)
Uncultured betaproteobacterium clone FTLM27 [AF529122]
Hydrogenophaga taniospiralis [AF078768]
Bacterium BH98-203 [AY928226]
Hydrogenophaga flava [AF078771]
OTUs B15-B16 (2 OTUs)
OTU B17
Uncultured betaproteobacterium clone AKYG1037 [AY921769]
Uncultured bacterium clone K4_3 [AY793658]
Burkholderia cepacia [U96927]
Uncultured betaproteobacterium clone MWH-UniP1 [AJ565421]
OTU B34
100
65
68
51
50
100
58
67
100
77
100
100
89
99
84
98
94
85
76
83
75
100
100
97
77
97
73
100
69
100
53
0.1

Figure 3-28: Phylogenetic tree showing relationships of Bacterial 16S rRNA gene sequences obtained from neat MFT samples to known β-proteobacterial sequences. Clones fall into clusters indicated on the tree by one representative. The tree was constructed from partial (~500 bp) 16S rRNA gene sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequence difference. The tree was rooted using *M. mazei* as an out-group.

A large group of OTUs (B20 to B28) appeared to be closely related to the genus *Thiobacillus* (95 to 98%) and three OTUs (B17 to B19) were more distantly related to *Thiobacillus* (<95%) (Figure 3-28). *Thiobacillus* oxidize elemental sulfur (S^0), hydrogen sulfide (H_2S), and thiosulfate ($S_2O_3^{2-}$) to sulfuric acid (Prescott et al., 1999). Although most species of *Thiobacillus* are aerobic, some, such as *T. denitrificans* and *T. thioparus*, can grow anaerobically by coupling the oxidation of sulfur to the reduction of nitrate to nitrogen gas (Prescott et al., 1999; Robertson and Kuenen, 2002). The largest numbers of these clones were found in the 6 to 20 m depths of MLSB (16 to 20% of the respective clone libraries, with lower numbers at MLSB 30 m and in all depths of WIP (2 to 9% of respective clone libraries).

Four OTUs (B29 to B32) were not closely related to any cultured species, but were related to several uncultured clones (Figure 3-28). Clone MFTT34 (AF796049; unpublished) was detected from borehole water from a South African gold mine. Clone 39-6 (AF351219) was found in coal-tar-waste contaminated soil (Bakermans and Madsen, 2002), so these microorganisms may be involved in the degradation of hydrocarbon contaminants. Clone SG2-153 (AY135933; unpublished) was detected in Lake Sapgyo, Korea. The largest proportion of this group of clones was found in the MLSB 30 m library, associating them with the oldest MFT in MLSB. This may mean that they were originally present in the MLSB area before tailings were deposited.

OTU B33 was related to two uncultured clones, AKYG1037 (AY921769) isolated from a soil metagenomics project, and clone K4-3 was found in lake sediment containing significant concentrations of the steroid trenbolone (Figure 3-28).

OTU B34 was related to *Burkholderia cepacia*, which is able to aerobically metabolize a wide range of organic molecules, and therefore is very important in the degradation of organic matter in nature (Prescott et al., 1999).

OTU B35 was distantly related to the genus *Ferribacterium*, and to clones isolated from a uranium-contaminated surface sediment (FB46-01;AY527762; unpublished) and clone KD1-23 (AY188296; unpublished), isolated from a deep sea sediment core (Figure 3-28).

OTU B36 was related to *Thauera phenylacetica* and the genus *Azoarcus* (Figure 3-28). Both of these genera contain facultatively aerobic species that are able to degrade aromatic compounds including toluene and benzoic acid, by the reduction of nitrate to nitrogen gas. They are commonly associated with root rhizosphere soil, and can also be isolated from sewage sludge or contaminated soil. *Thauera* spp. are also able to degrade aromatic compounds under denitrifying conditions (Shapleigh, 2000).

3.3.8 γ-proteobacteria

The libraries constructed from 10 and 20 m depths of MLSB, and the 6 m depth of WIP contained the highest numbers of γ-proteobacteria (10-20% of the respective libraries). γ-Proteobacteria are a large group of Gram negative bacteria with a wide range of physiological characteristics. However, the majority of OTUs related to the γ-proteobacteria were not closely related to any isolated species (Figure 3-29), which makes it difficult to speculate on the role of the γ-proteobacteria from the tailings.

One large group of γ-proteobacterium clones (OTUs B42 to B51) was related to several uncultured clones from other studies (Figure 3-29). Most of these clones were isolated from contaminated sites. Clone IAFR510 (AF270959) was isolated from pentachlorophenol-degrading soil (Beauleiu et al., 2000). Clones PAH-Feed-52 and PYR10d11 (DQ123671 and DQ213783; unpublished) were isolated in the same study from soils contaminated with polycyclic aromatic hydrocarbons. Clone LTUG0956 (AY144261) was found in petroleum contaminated soil (Kaplan and Kitts, 2004). The relationship to hydrocarbon-degrading clones suggests that these clones may be involved in degrading residual bitumen present in the tailings. In contrast, clone CCDS_DF1080_B22 (AY820699) was isolated from drilling fluid in a study of ultra-high-pressure rocks in China (Zhang et al., 2005). The highest numbers of these clones occurred in the MLSB 10 m and 20 m libraries (9.6 and 13.8%, respectively).

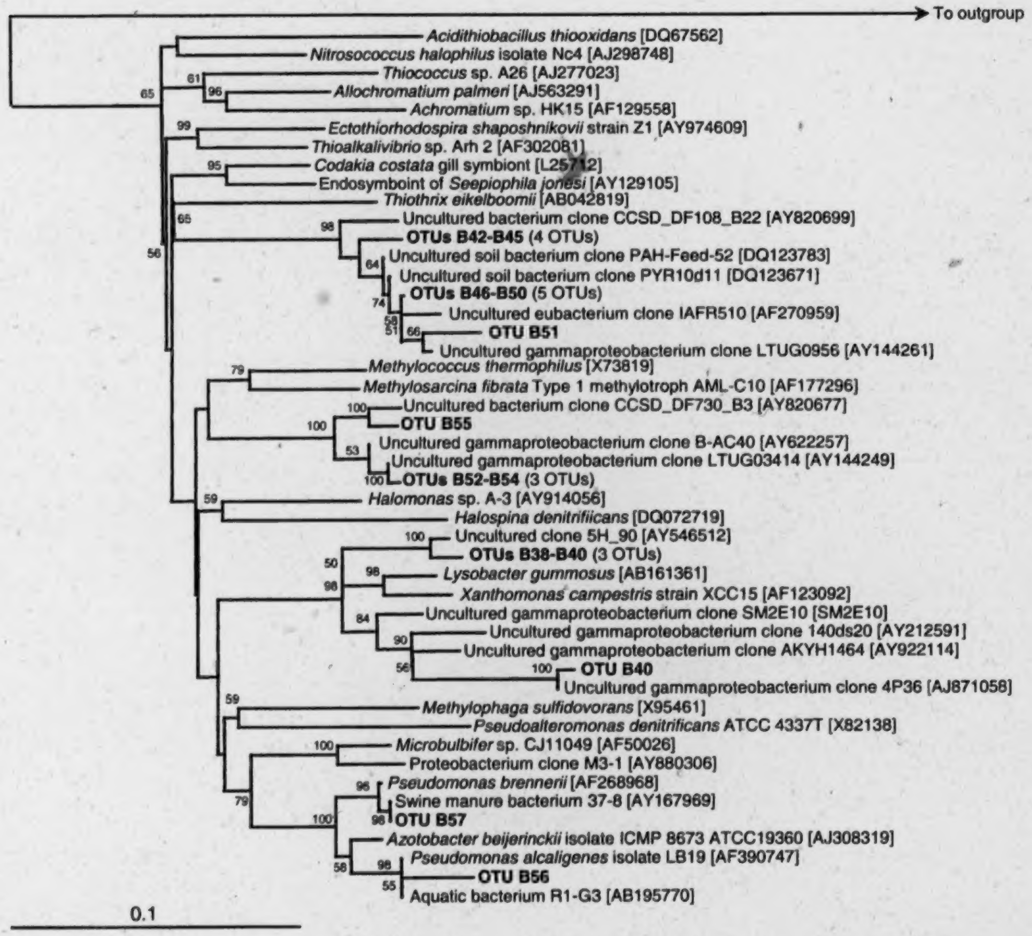
To outgroup
Acidithiobacillus thiooxidans [DQ67562]
Nitrosococcus halophilus isolate Nc4 [AJ298748]
Thiococcus sp. A26 [AJ277023]
Allochromatium palmeri [AJ563291]
Achromatium sp. HK15 [AF129558]
Ectothiorhodospira shaposhnikovii strain Z1 [AY974609]
Thioalkalivibrio sp. Arh 2 [AF302081]
Codakia costata gill symbiont [L25712]
Endosymboint of Seepiophila jonesi [AY129105]
Thiothrix eikelboomii [AB042819]
Uncultured bacterium clone CCSD_DF108_B22 [AY820699]
OTUs B42-B45 (4 OTUs)
Uncultured soil bacterium clone PAH-Feed-52 [DQ123783]
Uncultured soil bacterium clone PYR10d11 [DQ123671]
OTUs B46-B50 (5 OTUs)
Uncultured eubacterium clone IAFR510 [AF270959]
OTU B51
Uncultured gammaproteobacterium clone LTUG0956 [AY144261]
Methylococcus thermophilus [X73819]
Methylosarcina fibrata Type 1 methylotroph AML-C10 [AF177296]
Uncultured bacterium clone CCSD_DF730_B3 [AY820677]
OTU B55
Uncultured gammaproteobacterium clone B-AC40 [AY622257]
Uncultured gammaproteobacterium clone LTUG03414 [AY144249]
OTUs B52-B54 (3 OTUs)
Halomonas sp. A-3 [AY914056]
Halospina denitrifiicans [DQ072719]
Uncultured clone 5H_90 [AY546512]
OTUs B38-B40 (3 OTUs)
Lysobacter gummosus [AB161361]
Xanthomonas campestris strain XCC15 [AF123092]
Uncultured gammaproteobacterium clone SM2E10 [SM2E10]
Uncultured gammaproteobacterium clone 140ds20 [AY212591]
Uncultured gammaproteobacterium clone AKYH1464 [AY922114]
OTU B40
Uncultured gammaproteobacterium clone 4P36 [AJ871058]
Methylophaga sulfidovorans [X95461]
Pseudoalteromonas denitrificans ATCC 4337T [X82138]
Microbulbifer sp. CJ11049 [AF50026]
Proteobacterium clone M3-1 [AY880306]
Pseudomonas brennerii [AF268968]
Swine manure bacterium 37-8 [AY167969]
OTU B57
Azotobacter beijerinckii isolate ICMP 8673 ATCC19360 [AJ308319]
Pseudomonas alcaligenes isolate LB19 [AF390747]
OTU B56
Aquatic bacterium R1-G3 [AB195770]
65
61
96
99
95
65
56
98
64
74
58
51
66
79
100
100
53
100
59
100
50
98
98
84
90
56
100
59
100
96
79
100
98
58
98
55
0.1

Figure 3-29: Phylogenetic tree showing relationships of Bacterial 16S rRNA gene sequences obtained from neat MFT samples to known γ-proteobacterial sequences. Clones fall into clusters indicated on the tree by one representative. The tree was constructed from partial (~500 bp) 16S rRNA gene sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequence difference. The tree was rooted using *M. mazei* as an out-group.

A third group of OTUs related to uncultured clones (B52 to B55) was most closely related to clone CCSD_DF730_B3 (AY820677) (Figure 3-29), isolated from drilling fluid in a study of ultra-high-pressure rocks (Zhang et al., 2005). Also, they were related to clone B-AC40 (AY622251), which was isolated from an acidic aquifer contaminated with uranium (Reardon et al., 2004). This suggests that these may be present in geological rock formations. Clone LTUG03414 (AY144249) was isolated from petroleum-contaminated soil (Kaplan 2004) which suggests that this group of clones may be able to degrade the hydrocarbons present in the MFT.

This group of OTUs was also more distantly related to *Methylosarcina* and *Methylococcus* spp. (Figure 3-29). Both of these genera are methylotrophic, using one-carbon compounds such as methane and methanol as their sole carbon and energy sources under aerobic or microaerophilic conditions. These microorganisms may be consuming the methane being constantly produced, although they require at least some oxygen to function.

A group of OTUs (B39 to B41) was not closely related to known species of γ-proteobacteria, but instead were most closely related to clone 5H_90 (AY546512) which was identified using stable isotope probing from a Transbaikal soda lake (Lin et al., 2004) (Figure 3-29). They were also related to clone 4P36 (AJ871058; unpublished) that was amplified from an oil-degrading consortium. This indicates that these bacteria may also be involved in degrading the residual hydrocarbons present in the MFT. These clones were also more distantly related to *Xanthomonas* and *Lysobacter* spp. *Xanthomonas* are closely related to *Pseudomonas*, and are aerobic soil-inhabiting microorganisms which are able to degrade a range of organic compounds (Reichenbach, 1999b). *Lysobacter* spp. are gliding soil-inhabiting bacteria which are ecologically important due to their production of exoenzymes and antibiotics (Reichenbach, 1999b). In nature they secrete lytic enzymes which kill other bacteria, providing the *Lysobacter* with organic molecules for growth. Most strains are aerobic, but some can grow by fermentation as well.

Two OTUs were found (B56 and B57) which are related to *Pseudomonas* and *Azotobacter* spp. (Figure 3-29). *Pseudomonas* are usually aerobic bacteria, although some species can use nitrate as an electron acceptor (Prescott et al., 1999). *Azotobacter* is commonly found in soil and water environments, is aerobic, and is able to fix atmospheric nitrogen.

3.3.9 δ-proteobacteria

OTUs related to the δ-proteobacteria were found in libraries constructed from both MLSB and WIP; however there were many more clones related to the δ-proteobacteria found in the libraries constructed from WIP (10 to 15% of WIP libraries, compared to 0 to 3% of MLSB libraries). As with the γ-proteobacteria, the clones do not cluster with known species (Figure 3-27), so it is difficult to speculate on their role in the tailings environment.

δ-Proteobacteria are made up of two distinct groups of bacteria. One group is made up of predators such as the myxobacteria and the bdellovibrios, and the other group is made up of SRB (Prescott et al., 1999). All 41 of the δ-proteobacterial clones isolated from the tailings ponds clustered with the SRB. The higher numbers of δ-proteobacteria in WIP correlated with the elevated levels of sulfate in WIP (20 to 75 mg/L) compared to MLSB (0.1 to 35 mg/L: Table 3-7). This suggests that the OTUs related to the δ-proteobacteria do in fact represent uncultivated species of SRB.

SRB are strictly anaerobic bacteria that reduce oxidized sulfur compounds to sulfide while oxidizing small organic molecules such as lactate, pyruvate, ethanol or fatty acids. They are very common in aquatic environments and are important in the cycling of sulfur and in hydrocarbon degradation in anaerobic environments (Madigan et al., 2000). SRB have also been shown to be able to activate *n*-alkanes by coupling the activation of the hydrocarbon with fumarate with the reduction of sulfate (Kropp et al., 2000).

A large group of OTUs (B58 to B63) were related to the uncultured clone ROMEm4sh208 (AY998127; unpublished) found in deep subsurface shale samples, and clone 1013-1-CG26 (AY532547; unpublished) detected in a uranium-contaminated aquifer (Figure 3-27). OTU B64 did not cluster with any other clones from this study and was only distantly related to an uncultured clone ML320-J28 (AY458255; unpublished), amplified from a hypersaline lake.

OTU B65 is interesting as it is fairly closely related to *Desulfocapsa thiozymogenes* (96%), which is a sulfate reducer (Rabus et al., 2000). Sulfate reduction can also be coupled with the oxidation of alcohols to fatty acids (Janssen et al., 1996). It is also related to an uncultured clone sipK108 (AJ307944) which was isolated from a sulfidic marsh (Moissl et al., 2002). This suggests that this microorganism may be involved in sulfate reduction in the tailings, and may also be involved in the oxidation of hydrocarbons in the tailings to smaller fatty acids.

Five OTUs (B66 to B70) were related to several uncultured clones, as well as more distantly related to members of the genus *Syntrophus* (Figure 3-27). Members of this genus participate in the syntrophic oxidation of organic molecules along with a hydrogen-scavenging methanogen. *Syntrophus gentianae* is known for its ability to syntrophically degrade benzoate with a hydrogenotrophic methanogen to remove the extra hydrogen (Kersters et al., 2003). These OTUs also clustered with uncultured clones identified from a deep well injection site (S15B-MN12; AJ583188; unpublished), a coal-tar-waste contaminated aquifer (36-11; AF351220; (Bakermans and Madsen, 2002)), a coal-contaminated area of forested wetland (FW99; AF523966; (Brofft et al., 2002)), the rhizosphere of *Camptotheca acuminata* (ga83; AY466856; unpublished), and a hydrocarbon- and chlorinated solvent-contaminated aquifer (WCHB1-12; AF050534; (Dojka et al., 1998)). This wide variety of environments from which related clones have been identified indicates that these microorganisms may be important in the degradation of organic compounds, and may be involved in hydrocarbon degradation in MFT.

Five OTUs (B72 to B75) were distantly related to an uncultured δ-proteobacterium (Figure 3-27) clone Hyd86-61 (AJ535249) which was identified from the sediment above a methane hydrate deposit (Knittel et al., 2003), and was suspected of being involved in the anaerobic oxidation of methane along with syntrophic relationships with the ANME-2 group bacteria.

One OTU (B76) as closely related to uncultured bacterium clone Qui4P2-36 (AJ518578) which was isolated from a eutrophic reservoir (Wobus et al., 2003) (Figure 3-27).

3.3.9.1 Chloroflexi

Two OTUs (B81 and B82) clustered with the Chloroflexi family of bacteria (Figure 3-27). These are filamentous anoxogenic photosynthetic bacteria which are physiologically very similar to the green sulfur bacteria (Hanada and Pierson, 2002). In addition, these OTUs are closely related to clones isolated from areas contaminated with chlorinated solvents. These OTUs are present in very low numbers in all of the clone libraries (Figure 3-24), so they may not have a significant role in the tailings environment. It is also unclear why there are photosynthetic bacteria present in the tailings, when clearly no sunlight could reach the depths of the MFT, unless their DNA was preserved in the deep MFT samples.

3.3.9.2 Clostridia and *Acholeplasma*

Nine OTUs clustered with the clostridia. Two OTUs (B100 and B101) fell into cluster XI (Figure 3-30), along with clostridial clones found in the Bacterial libraries constructed from the columns. These are general fermenters of organic matter, and may be involved in breaking down larger organic molecules into the smaller fatty acids and alcohols which are used by the methanogens and acetogens. Two other OTUs (B113 and B114) clustered with a toluene-degrading methanogenic consortium bacterium clone, and therefore may be involved in degrading aromatic hydrocarbons present in the tailings. The rest of the OTUs did not cluster closely with any known species or bacterial clones, so their role in the tailings environment is unclear.

Low numbers of clones related to *Acholeplasma* species were present (Figure 3-30), with the highest occurrence in the MLSB 6 m library (5.4%, Figure 3-24, Figure 3-25). *Acholeplasma* are facultatively aerobic members of the family Mollicutes, and are characterized by their total lack of a cell wall (Madigan et al., 2000). Again, because of their low occurrence, they are not expected to play a significant role in nutrient cycling in the tailings environment.

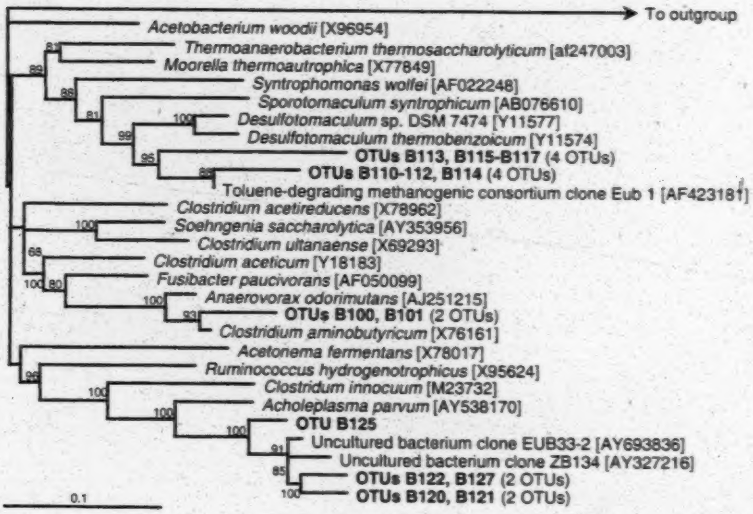
To outgroup
Acetobacterium woodii [X96954]
Thermoanaerobacterium thermosaccharolyticum [af247003]
Moorella thermoautrophica [X77849]
Syntrophomonas wolfei [AF022248]
Sporotomaculum syntrophicum [AB076610]
Desulfotomaculum sp. DSM 7474 [Y11577]
Desulfotomaculum thermobenzoicum [Y11574]
OTUs B113, B115-B117 (4 OTUs)
OTUs B110-112, B114 (4 OTUs)
Toluene-degrading methanogenic consortium clone Eub 1 [AF423181]
Clostridium acetireducens [X78962]
Soehngenia saccharolytica [AY353956]
Clostridium ultanaense [X69293]
Clostridium aceticum [Y18183]
Fusibacter paucivorans [AF050099]
Anaerovorax odorimutans [AJ251215]
OTUs B100, B101 (2 OTUs)
Clostridium aminobutyricum [X76161]
Acetonema fermentans [X78017]
Ruminococcus hydrogenotrophicus [X95624]
Clostridum innocuum [M23732]
Acholeplasma parvum [AY538170]
OTU B125
Uncultured bacterium clone EUB33-2 [AY693836]
Uncultured bacterium clone ZB134 [AY327216]
OTUs B122, B127 (2 OTUs)
OTUs B120, B121 (2 OTUs)
81
89
86
81
100
99
95
88
100
68
100
80
100
93
96
100
100
100
91
85
100
0.1

Figure 3-30: Phylogenetic tree showing relationships of Bacterial 16S rRNA gene sequences obtained from neat MFT samples to known clostridial sequences. Clones fall into clusters indicated on the tree by one representative. The tree was constructed from partial (~500 bp) 16S rRNA gene sequences using a distance matrix based Neighbor-Joining method. Bootstrap values were calculated from 100 replicates and are indicated on the branches. Bootstrap values less than 50% are not shown. The scale bar represents 10% sequence difference. The tree was rooted using *M. mazei* as an out-group.

3.3.10 Summary

Most of the Archaeal clones in the neat MFT were closely related to acetoclastic *Methanosaeta* spp., suggesting that acetoclastic methane production is the dominant pathway in situ in tailings settling basins.

The majority of the bacterial clones detected in the neat MFT clustered with the proteobacteria, and most were related to uncultured clones identified in hydrocarbon- and solvent-contaminated soils and aquifers. Many of the closely related bacteria are commonly found in anaerobic environments, and it is not surprising to find them in the anaerobic tailings. Because many of these clones and related species have been detected in various hydrocarbon- and solvent-contaminated sediments, they may have the ability to degrade hydrocarbons.

Large numbers of clones clustered with the sulfate-reducing members of the δ-proteobacteria, and were found in greater numbers in WIP which contains a higher concentration of sulfate than does MLSB. These SRB have the ability to degrade organic molecules and some can degrade hydrocarbons. Some members such as *Syntrophus* can participate in syntrophic oxidation of fatty acids with a methanogenic partner. We saw evidence of this mechanism of methane production acting in the methanogenic tailings cultures, where acetate did not appear to be directly used by the methanogenic species, but may have been first oxidized by an acetate-oxidizing syntroph (Section 3.2.1).

Some clostridial species were found in the tailings, but in lower proportions than in the acetate-amended tailings columns. This may indicate that when the tailings were amended with acetate and incubated at room temperature, the clostridia had a competitive advantage and were able to out-compete the proteobacterial species, which were present in much higher numbers in the neat MFT.

A large number of OTUs were related to aerobic bacteria. This was unexpected, because the tailings environment is highly anoxic. Perhaps the species represented by the clones are distantly enough related to the known aerobes that they are able to carry out fermentation or anaerobic respiration, and thus are able to survive in the anaerobic environment of the tailings. A few clones clustering with photosynthetic bacteria, such as the Chloroflexi, were found. The role of these microorganisms is not clear, as there will be no sunlight reaching even the shallow depths of the MFT. There are two explanations for the presence of aerobic and photosynthetic microorganisms in the deep MFT samples. The tailings samples were not taken aseptically, so there is a possibility that the samples were contaminated with microorganisms from the aerobic zones of the surface water. Also, microorganisms living in the surface water would eventually be carried into the MFT as detritus, and since the clone library technique only detects DNA and not viable microorganisms, they may not actually be living in the tailings but rather above them.

Interestingly, most of the Bacterial 16S rRNA gene sequences detected in the neat MFT were not closely related to known species, but were most closely related to uncultured clones. This is in contrast to the Archaeal 16S rRNA gene sequences, most of which were closely (98 to 99%) similar to isolated species. The reason for this is unknown, however it may be because the Bacterial species which we detected represent microorganisms which are difficult to culture, therefore are usually detected by DNA sequences analysis rather than by culture. Another reason may be that no on has attempted to culture these microorganisms before. Now that there are clues for their existence through 16S rRNA gene sequence analysis, methods for their cultivation can be developed and attempts to culture them can be made.

3.3.10.1 Clone library coverage

Accumulation curves were constructed for Archaeal and Bacterial clone libraries. Most of the accumulation curves for the Archaeal libraries (Figure 3-31) appear to plateau, so enough clones have probably been sampled to accurately represent the diversity present in the samples. This is also supported by the high coverage values for these clone libraries (Table 3-10). All of the Archaeal clone libraries have coverage values above 0.85.

The high coverage of the Archaeal clone libraries reflects the relatively low species richness values, none of which are above 2.20. In comparison, the species richness values for the Bacterial samples are relatively high, all above 3.40, and roughly correlate with the coverage of the libraries.

Table 3-10: Coverage values for Archaeal and Bacterial libraries constructed from MLSB and WIP tailings. Coverage was calculated using Equation 1-6, and Species Richness values were calculated with Equation 1-8.

	Coverage		**Species Richness**	
Sample	**Archaeal**	**Bacterial**	**Archaeal**	**Bacterial**
MLSB 6 m	0.98	0.47	1.64	3.74
MLSB 10 m	0.91	0.70	1.77	3.62
MLSB 20 m	0.95	0.66	1.16	3.73
MLSB 30 m	0.88	0.55	1.93	3.44
WIP 6 m	0.85	0.56	2.14	3.93
WIP 10 m	0.92	0.60	1.39	3.63
WIP 20 m	0.92	0.36	2.13	3.84

The Bacterial clone libraries constructed from the tailings were not big enough to accurately represent the diversity present in the samples. The accumulation curves shown in Figure 3-32 do not plateau, and several (Figure 3-32 C and E) are still increasing linearly, indicating that sampling more clones from these libraries would almost certainly result in the discovery of new OTUs and therefore greater diversity. The coverage values (Table 3-10) confirm that the clone libraries were not big enough to represent the diversity. Most of the coverage values are between 0.35 and 0.55, with the MLSB 10 m library having the highest value of 0.70. This library should have the highest coverage value, as approximately twice as many clones were picked for this library; however it was still not enough to provide adequate coverage of the diversity. The bacterial diversity present in the tailings was surprising, and perhaps further examination of this ecosystem should use a less labor-intensive community fingerprinting technique, such as DGGE or T-RFLP.

Even though the environment was not exhaustively sampled, very interesting results were obtained and provide a good starting point for further investigation.

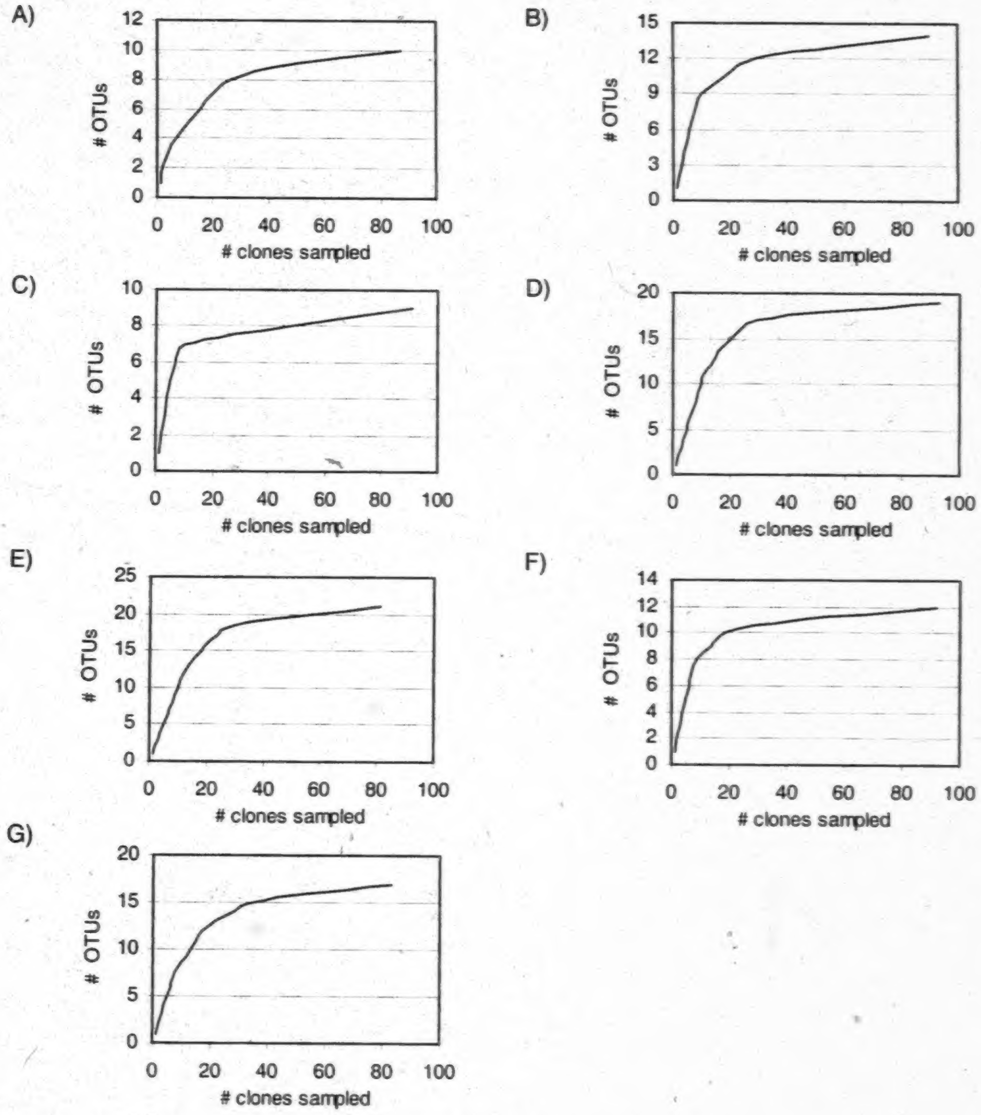

Figure 3-31: Accumulation curves for Archaeal clone libraries constructed from MLSB and WIP tailings. A) MLSB 6 m; B) MLSB 10 m; C) MLSB 20 m; D) MLSB 30 m; E) WIP 6 m; F) WIP 10 m; G) WIP 20 m.

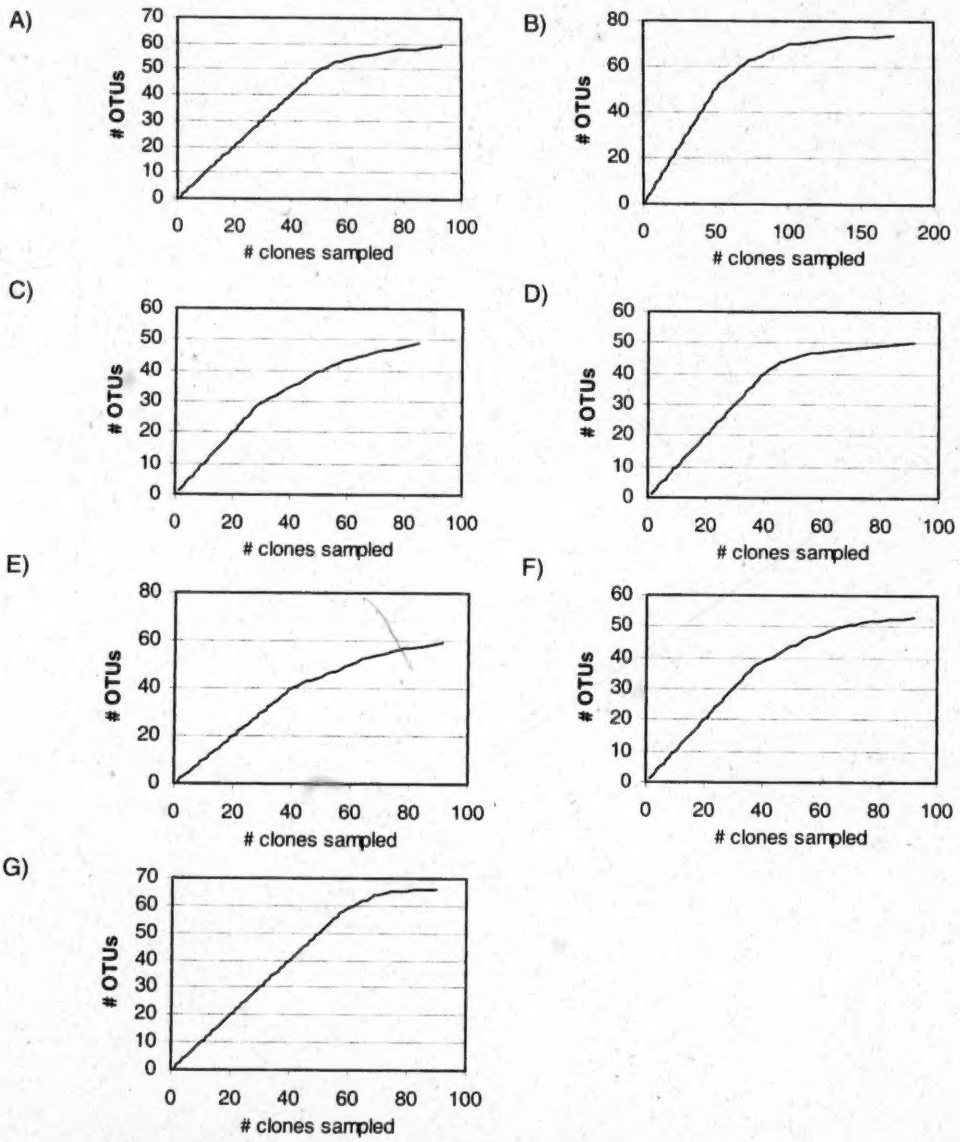

Figure 3-32: Accumulation curves for Bacterial clone libraries constructed from MLSB and WIP tailings. A) MLSB 6 m; B) MLSB 10 m; C) MLSB 20 m; D) MLSB 30 m; E) WIP 6 m; F) WIP 10 m; G) WIP 20 m.

3.4 Oil Sands Ore Analysis

After identifying the major methanogenic and many Bacterial species present in neat MFT from MLSB and WIP, the next objective was to determine if the methanogenic populations came from the oil sands ore. If methanogens could survive the extraction process, they would be deposited into the tailings ponds. Providing there is a suitable environment for their growth, they could then initiate methane production.

Four oil sands ore samples were obtained from Syncrude's North Mine site. These samples differed in percent fines content, which is important because microbes would be assumed to associate with the solids present in the oil sands. Some physical characteristics of the oil sands ore samples are shown in Table 3-11.

Table 3-11: Bitumen and fines content of four oil sands ore samples taken from the Syncrude North Mine (data provided by Syncrude).

Sample #	Bitumen (%)	Fines (%) (<22 μm)
1	12.2	19
2	12.1	15
3	8.9	27.2
4	8.4	23.3

3.4.1 Enumeration of Methanogens and SRB

Methanogenic and SRB MPN culture tubes inoculated with the fine tails component of the oil sands ores were all negative even after 2 months of incubation, indicating that there are no or very low numbers of viable methanogens and SRB present. However, this analysis could be repeated using a larger volume of ore as inoculum to compensate for the low numbers of microorganisms present in the ore samples.

3.4.2 Optimization of DNA extraction from the Oil Sands Ore

The FastSPIN DNA Extraction kit was used in small scale extractions to obtain genomic DNA from the oil sands. A positive control, containing just MPN culture, and an oil sands ore sample seeded with MPN culture, and two different forms of oil sands ore were processed. Oil sand sample #3 was chosen because it had the highest percent fines content, which presumably will be associated with any microorganisms present in the oil sands. Each of the extracted samples was used as the template for PCR amplification of Bacterial 16S rRNA gene fragments (Figure 3-33). 16S rRNA genes could be amplified from the positive control MPN culture, as well as from the ore sample seeded with 0.1 mL of MPN culture. This shows that it is possible to remove any potential inhibitors such as fine clay particles or components of bitumen from the oil sands samples and obtain pure DNA. However, no amplification product was seen from the extractions of either the fine tails or the settled oil sand, indicating that the numbers of microorganisms in the oil sands ores are too low to be detected using this PCR method.

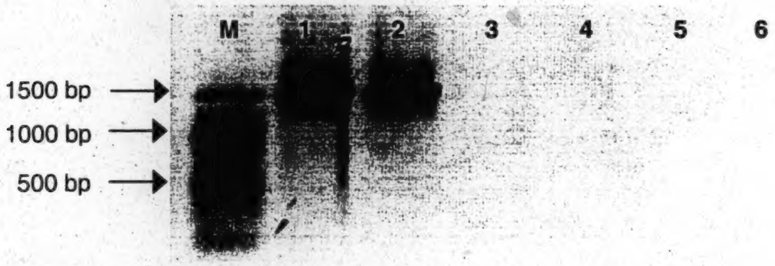

Figure 3-33: Amplification of Bacterial 16S rRNA genes from DNA extracted from oil sands ore samples using a small-scale extraction process (expected size ~1500 bp). M- molecular weight marker (sizes indicated are bp); Lane 1: MPN culture (positive control); Lane 2: Oil sands ore seeded with MPN culture; Lane 3: Oil sands ore sample #3 (fine tails); Lane 4: Oil sands ore sample #3 (sand and bitumen); Lane 5: Negative control for DNA extraction; Lane 6: Negative control for PCR.

Because the FastSPIN DNA extraction kit can only accommodate up to 0.5 g of sample, and this does not appear to contain enough DNA to amplify by PCR, the PowerMax™ Soil DNA Isolation Kit (Mo Bio Laboratories) was used, which can process up to 10 g of sample. Four samples were extracted: a) negative control (10 mL of sterile water), b) 10 mL of suspended oil sand in pyrophosphate buffer + 0.5 mL of MPN culture, c) 10 mL of suspended oil sands fines in pyrophosphate buffer, and d) 10 g of whole oil sand (not suspended in buffer). The quality and quantity of extracted DNA was assessed by PCR amplification using either Archaeal or Bacterial 16S rRNA gene primers. PCR amplification with Archaeal primers yielded no product for any of the samples (data not shown).

The results of the PCR amplification using the Bacterial 16S rRNA gene primers are shown in Figure 3-34. No product was seen for the negative extraction, indicating contamination had not likely occurred. Both extractions of suspended fine particles in the oil sands resulted in amplifiable DNA template, shown in lanes 7-16 in Figure 3-34. Lanes 15 and 16 have no visible product, indicating that the DNA concentration is so high that using 5 and 10 µL of DNA template has overloaded the PCR reaction, or has introduced high concentrations of inhibitory compounds. When 10 µL of the DNA extracted from the neat oil sand sample was used in the PCR reaction, it resulted in only a small amount of amplified 16S rRNA gene (lane 21, Figure 3-34). This indicates that suspending the oil sand in a buffer to separate the fine tails allows for more DNA to be extracted from the oil sand.

Because there was no PCR product observed when the Archaeal 16S rRNA gene primers were used, this may indicate that the number of *Archaea* in the oil sands ore is too low to be detected by this method, or that DNA from these microorganisms is more susceptible to PCR inhibition by compounds in the oil sand. Because the seeded oil sand containing 0.5 mL MPN culture did not provide an amplification product with the Archaeal primers but did amplify with Bacterial primers, this indicates that there may be selective inhibition of Archaea. However, it is promising that it is possible to obtain amplifiable Bacterial DNA from the oil sands, and this is an area which will be explored further. Future studies will involve identifying Bacterial species in the ore, as well as optimizing the amplification of Archaeal 16S rRNA genes from the oil sands ore.

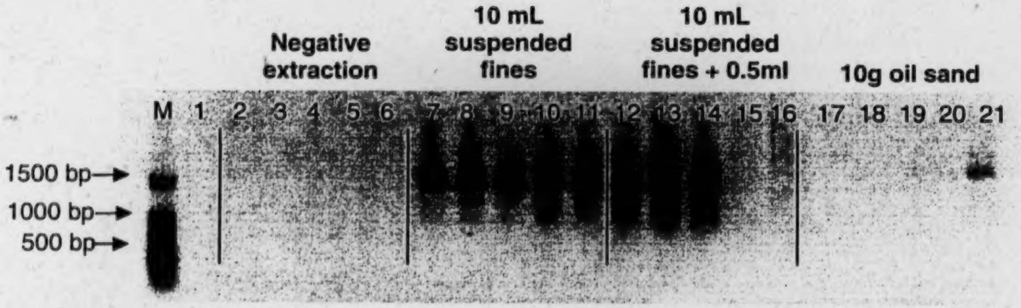
10 mL
Negative extraction
10 mL suspended fines
10 mL suspended fines + 0.5ml
10g oil sand
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
1500 bp
1000 bp
500 bp

Figure 3-34: Amplification of Bacterial 16S rRNA genes from DNA extracted from oil sands ore samples using a large-scale extraction process (expected size ~ 1500 bp). Lane M: 100 bp molecular weight DNA ladder; Lane 1: Negative PCR control. Lanes 2-6: Negative extraction (10 mL sterile water); Lanes 7-11: 10 mL suspended oil sands fines in pyrophosphate buffer; Lanes 12-16: 10 mL suspended oil sands fines in pyrophosphate buffer + 1.0 mL MPN culture; Lanes 17-21: 10 g neat oil sand; Lanes 2, 7, 12, 17: 0.5 µL DNA template; Lanes 3, 8, 13, 18: 1.0 µL DNA template; Lanes 4, 9, 14, 19: 2 µL DNA template; Lanes 5, 10, 15, 20: 5 µL DNA template; Lanes 6, 11, 16, 21: 10 µL DNA template.

3.5 Summary of microbial processes occurring in the MFT

3.5.1 Methanogenic species in laboratory column studies and neat MFT

There is a marked difference between the Archaeal populations in the libraries constructed from MPN cultures (containing acetate) incubated at room temperature for a long period of time, and those from neat tailings samples taken from MLSB and WIP and stored at 4°C for several months. The acetate-amended cultures have a dominant group of sequences related to hydrogenotrophic methanogens, with a smaller proportion of sequences related to acetoclastic methanogens. However, the libraries constructed from MFT taken from the tailings ponds and analyzed without prior culture have an abundance of sequences related to acetoclastic methanogens, indicating that these may be the dominant species present in the tailings ponds.

The difference in methanogenic populations may either be due to differences in acetate concentration, or the incubation temperature of the cultures. In the column studies, MPN cultures from acetate-amended and unamended tailings were compared, and although there were some small differences in the sequences found in the initial-time and final-time samples, there were no marked changes in the populations that could be correlated with acetate amendment in either the highest or lowest MPN dilution tubes. All of the samples taken from the acetate-amended or unamended columns were incubated in MPN culture medium containing acetate as a carbon source, and this could have shifted the methanogenic populations in favor of those which could more readily use the acetate, or could have enriched for homoacetogenic bacteria which produced acetate for the methanogens. However, since hydrogenotrophic methanogens were the dominant clones in all of the samples, I think the shifts in population were influenced more by the incubation temperature of the samples. In the column studies, the tailings were equilibrated at 4°C for approximately three months, and then transferred to 25°C for one month. Samples were taken before and after the incubation period. These samples were then used to inoculate MPN culture tubes, which were incubated at room temperature for 30 d, and then stored at 4°C for a number of months. This incubation at higher temperature may have allowed Bacteria capable of syntrophically oxidizing acetate to grow, producing H_2+CO_2 for the hydrogenotrophic methanogens. Indeed, the majority of Bacterial sequences found in the libraries constructed from the cultured MFT were related to the clostridia, a group containing many homoacetogens and known syntrophs.

In contrast, MFT samples were taken from MLSB and WIP, chemically characterized, and then stored at 4°C for several months until the DNA was extracted. The long periods of incubation of the column samples at room temperature will certainly have caused the methanogenic populations to shift due to thermodynamic considerations, discussed below, which will have an effect on the pathway of methane generation. The dominant OTUs in the libraries constructed from the neat tailings were related to the genus *Methanosaeta*, an obligate acetoclastic methanogen. There were very few OTUs related to hydrogenotrophic methanogens, indicating that most of the carbon flow in the MFT is going through acetate, not CO_2. This supports the hypothesis that acetogens may be responsible for the breakdown of hydrocarbon molecules in the tailings, providing acetate for methanogenesis.

The appearance of Crenarchaeota in the neat MFT libraries is interesting, although perhaps not too surprising. This group of Archaea consists of mostly uncultured clones detected from mainly chemolithotrophic environments, indicating that these microorganisms may be utilizing inorganic molecules, such as sulfur and H_2, for energy. The detection of these sequences only in the deepest samples from MLSB and WIP indicates that they are living in the oldest, and most depleted areas of the MFT. They would be out-competed when there are significant amounts of substrates available for the other microorganisms in the tailings, and only when these substrates are depleted can they go on to survive on the inorganic components of the MFT.

3.5.2 Thermodynamic considerations

There are four main groups of microorganisms active in anaerobic, methanogenic environments. The first are fermentative bacteria, which convert organic polymers and monomers to H_2, alcohols, and small fatty acids like acetate and propionate. The second are homoacetogens, which ferment organic matter producing only acetate as a byproduct, and can also grow autotrophically by reducing CO_2 with H_2. The third and fourth are types of methanogenic *Archaea*, which produce methane either using acetate (acetoclastic), or H_2+CO_2 (hydrogenotrophic) as substrates. The various proportions in which these groups are present are affected by variables in the environment, including pH, temperature, redox potential, substrate availability, and nutrient concentration (Fey and Conrad, 2000).

Thermodynamics have a large effect on determining which populations will dominate in an environment, and therefore will determine the pathway of carbon flow through an environment. The microorganism which carries out the most thermodynamically favorable reaction, thereby obtaining the most energy per substrate molecule, will dominate when there is competition for that substrate.

Different methanogenic populations in samples incubated at different temperatures have been observed in samples from various environments, including rice paddy soil (Chin and Conrad, 1995; Lueders et al., 2004), lake sediment (Svensson, 1984; Schulz and Conrad, 1996; Schulz et al., 1997; Lay et al., 1998), river marshland (Wagner and Pfeiffer, 1997), wetland peat bog (Kotsyurbenko et al., 2004), and enrichment cultures (Chin et al., 1999). All of these environments had a dominance of acetoclastic methanogenic species at low temperature (<15°C) and higher numbers of hydrogenotrophic methanogens at higher temperatures (>20°C).

The first step in the aerobic degradation of organic matter is fermentation to fatty acids and alcohols, which releases most of the available Gibbs free energy for use by the fermenting bacteria (Conrad, 1999). The second step is the degradation of these fatty acids and alcohols into acetate and CO_2. This can be done by syntrophic bacteria which release acetate, CO_2 and H_2 as byproducts. This process is only energetically favorable if the H_2 concentration is kept very low by a hydrogenotrophic methanogen. Less than half of the available free energy from the organic molecules is left for syntrophic oxidation, and this must be shared between the syntroph and the methanogen. The other pathway by which fatty acids are converted to acetate is performed by homoacetogens, which do this without producing H_2, thereby eliminating the need for a hydrogen-consuming methanogen. The acetate can then be used by an acetoclastic methanogen, and although less energy is recovered by acetoclastic than by hydrogenotrophic methanogenesis, it does not have to be shared with a syntroph.

Which of these processes will convert fatty acids and alcohols to methane is largely determined by thermodynamics. Hydrogen-producing fermentation of fatty acids becomes less favorable as temperature decreases (Kotsyurbenko et al., 2004) (see Section 1.2.1), so at low temperature homoacetogenic fermentation will dominate. The acetate produced by homoacetogens can then be used by acetoclastic methanogens, which is the main reason carbon flow tends to go through acetate to methane at low temperature.

H_2 is a very important intermediate in all anaerobic environments, and tends to have a very low concentration due to high turnover. Competition for hydrogen will therefore determine what microorganisms will dominate in an environment. The better a microorganism is able to take up H_2, the more competitive it will be. The two major consumers of H_2 in a methanogenic environment are homoacetogens and hydrogenotrophic methanogens. Temperature has a major effect on the competitiveness of these two groups of microorganism for H_2.

Microorganisms have different concentration thresholds for H_2 metabolism, that is, there is a minimum concentration of H_2 required for the microorganism to utilize it (Conrad, 1999). The threshold value is related to the minimum amount of energy that is required to synthesize 1/3 ATP molecule. A microorganism with a lower H_2 threshold will have an advantage because it can grow even in low H_2 concentrations, and is better able to compete for H_2 when it is limiting. It has been shown that homoacetogens have a higher H_2 threshold than do methanogens, meaning methanogens can utilize a lower H_2 concentration than homoacetogens (Kotsyurbenko et al., 2001; Kotsyurbenko, 2005). This should give hydrogenotrophic methanogens a competitive advantage over homoacetogens; however, this is not always the case in the environment.

Homoacetogens seem to be better adapted to growth at low temperatures (Kotsyurbenko, 2005), and also have a wide substrate range, which is probably why they are seen to dominate in many cold-temperature environments. In addition, due to their versatile metabolic capabilities (Drake and Danial, 1997), homoacetogens can switch to homoacetogenic fermentation at low temperatures, degrading larger organic molecules to acetate, instead of choosing to compete with hydrogenotrophic methanogens for H_2. This provides the acetate for acetoclastic methanogenesis, which explains why it is the dominant process in cold environments. Homoacetogens also have a much higher growth rate at low temperatures compared to methanogens (Kotsyurbenko et al., 1996).

Methanosaeta spp. were the dominant methanogens present in the neat MFT samples. This same dominance of *Methanosaeta* spp. over *Methanosarcina* spp, which are both acetoclastic methanogens, has also been observed in rice field soil (Fey and Conrad, 2000). *Methanosaeta* spp. have lower K_m values and thresholds for acetate, so are better able to compete for substrate, and can grow faster than *Methanosarcina* at low temperature (Fey and Conrad, 2000).

The effect of temperature on the pathway of methane generation in the tailings ponds is a very relevant question. The pathway of carbon flow will greatly influence the relative rate of methane generation. Because temperatures in MLSB can vary from 60°C in areas where fresh tailings are entering the pond, to 10 to 20°C in areas more removed from the outflow (Table 3-7), the mechanism by which methane is generated will almost certainly be different in various areas of the pond. Temperature also varies with MFT depth, with older, deeper MFT having a higher temperature than newer MFT at shallower depths (Table 3-7). Nutrient concentration will also change with depth, with the deeper MFT being more depleted in readily metabolized substrates. This heterogeneous situation is not ideal for formulating a simplistic strategy for increasing the methane generation, or to aim for consistent densification rates across the pond. Different amendment strategies may have to be developed for different temperature ranges, in order to optimize methane generation in all areas of the tailings pond.

Some sulfate reducers can function both as homoacetogens and sulfate reducers. It has been shown that all known syntrophic propionate reducers can also reduce sulfate (Conrad, 1999). Due to competition for H_2, and the fact that sulfate reduction allows a microorganism to have a lower H_2 threshold, this would decrease the H_2 in an environment to a point where the hydrogenotrophic methanogens can no utilize the H_2 (Conrad, 1999). Some sulfate reducers will also degrade acetate to CO_2 (Madigan et al., 2000). Methanogens and SRB cannot grow together in close proximity when H_2 concentrations are limiting because SRB decrease the H_2 concentrations below the H_2-threshold for the methanogens. SRB also have a higher growth rate than methanogens, so the populations are able to take over when sulfate is available (Conrad, 1999). Acetoclastic methanogenesis also does not occur in this situation, possibly because the SRB have a lower acetate threshold than acetoclastic methanogens, therefore inhibiting the acetoclasts in the same manner as for the hydrogenotrophic methanogenesis (Conrad, 1999). This is just speculation, and has not yet been shown in any studies.

3.5.3 Working hypothesis explaining methane production in neat MFT

The first step in degradation of organic matter in the tailings may be the initial attack on hydrocarbon molecules (Figure 3-35). SRB may be involved in the initial oxidation of hydrocarbons in the naphtha or the residual bitumen to produce organic acids and alcohols. It has previously been demonstrated that SRB are able to oxidize *n*-alkanes with the reduction of sulfate, producing methyl-branched fatty acids such as (1-methyltetradecyl)succinate and (1-

methylpentadecyl)succinate (Cravo-Laureau et al., 2005). I was able to detect sequences related to the δ-proteobacteria and known SRB in the clone libraries constructed from the tailings. Iron-reducing bacteria such as *R. ferrireducens* may also be involved in the oxidation and degradation of hydrocarbons, based on iron or nitrate reduction (Finneran et al., 2003; Jahn et al., 2005).

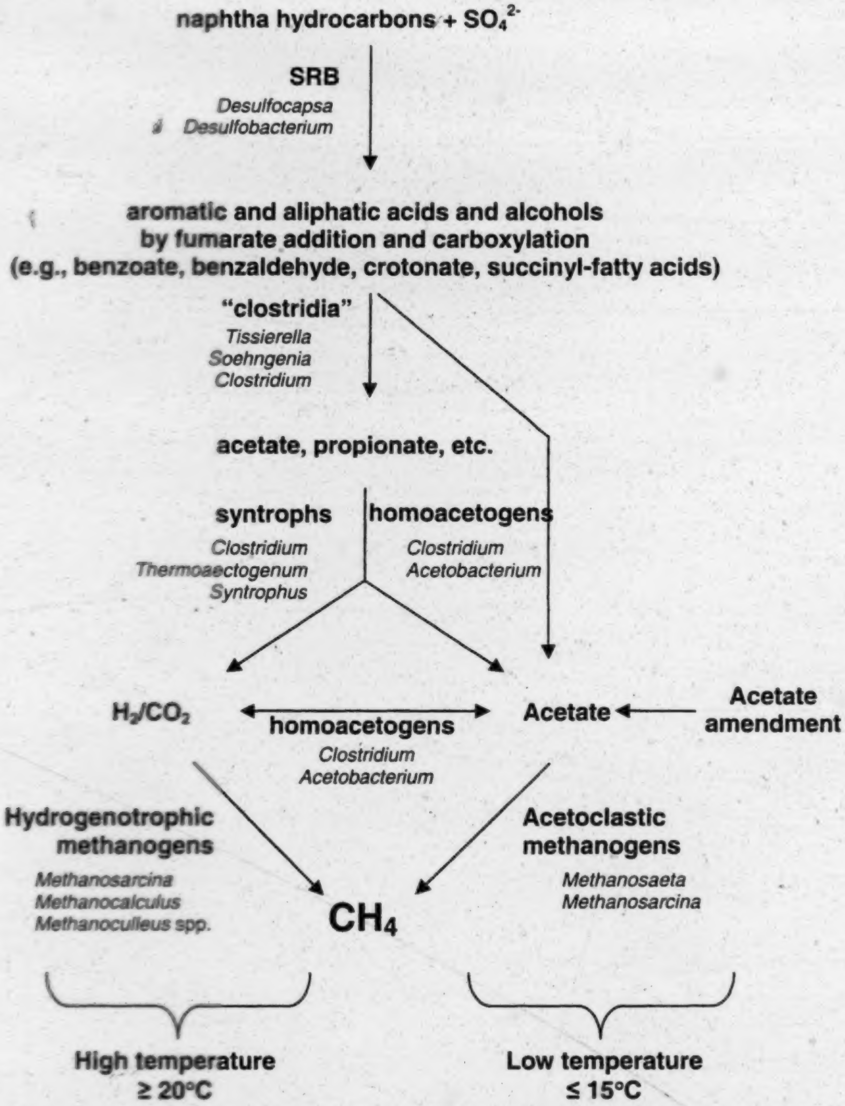
naphtha hydrocarbons + SO4 2-
SRB
Desulfocapsa
Desulfobacterium
aromatic and aliphatic acids and alcohols
by fumarate addition and carboxylation
(e.g., benzoate, benzaldehyde, crotonate, succinyl-fatty acids)
"clostridia"
Tissierella
Soehngenia
Clostridium
acetate, propionate, etc.
syntrophs
Clostridium
Thermoaectogenum
Syntrophus
homoacetogens
Clostridium
Acetobacterium
H2/CO2
homoacetogens
Clostridium
Acetobacterium
Acetate
Acetate amendment
Hydrogenotrophic methanogens
Methanosarcina
Methanocalculus
Methanoculleus spp.
CH4
Acetoclastic methanogens
Methanosaeta
Methanosarcina
High temperature
≥ 20°C
Low temperature
≤ 15°C

Figure 3-35: Possible scheme for methane production in the MFT. Adapted from Conrad (1999). Genera indicated were all detected in 16S rRNA gene clone libraries constructed from cultured or uncultured MFT.

After the hydrocarbons have been initially oxidized, they may be available for fermentation by other heterotrophic bacteria, eventually producing acetate and CO_2. Members of the clostridia are able to degrade organic acids and alcohols to smaller organic molecules (Hippe et al., 1999). We have detected the presence of *Tissierella*, *Soehngenia*, and *Clostridium* spp. in the bacterial clone libraries, all of which are members of the clostridia and are reported to break down organic molecules to produce small fatty acids (acetate, propionate), CO_2 and H_2. Homoacetogens can also degrade larger organic molecules to acetate. I did not detect any OTUs related to known acetogens in the MFT, but they must be present in MLSB because they were enriched and subsequently detected in the methanogenic cultures inoculated with the column tailings. Homoacetogenic clones might also have been detected in the neat tailings libraries if all of the clones had been sequenced rather than just the abundant OTUs.

The small organic acids produced from the fermentation step are then degraded by homoacetogens and acetoclastic methanogens. I detected a large number of acetoclastic methanogens, specifically *Methanosaeta* spp. in the libraries constructed from the neat tailings, which supports the hypothesis that homoacetogenesis and acetoclastic methanogenesis dominate at the low temperatures in most of MLSB and WIP. Some SRB have demonstrated homoacetogenic activity (Conrad, 1999), and these species may be supporting methane generation through acetogenesis, especially at depths in MLSB where the concentrations of sulfate are depleted (Table 3-7).

Fermentation products will include acetate, CO_2 and H_2, which are substrates for methanogens and homoacetogens. At low temperature (< 15°C) in areas further from contemporary outflow or at shallow depth in MLSB, homoacetogens are better able to compete for the limiting amounts of H_2, and most of the flow of carbon will go through acetate, which is a substrate for acetoclastic methanogens. At higher temperatures (e.g. in MFT nearer to the outflow or at greater depth), syntrophic bacteria will oxidize fatty acids to CO_2 and H_2, which is made possible by the removal of H_2 by hydrogenotrophic methanogens. However, when tailings are removed from the ponds and incubated in laboratory conditions at room temperature, the pathway shifts from mainly acetoclastic to mainly hydrogenotrophic methanogenesis.

Regardless of the specific pathway of methane production in the oil sands tailings, both methane and CO_2 are produced by the methanogenic communities. Both of these products may be involved in the increase in tailings densification. Methane gas is largely insoluble in water, and accumulates in bubbles within the tailings. Once the bubbles reach a critical volume, they escape through the tailings matrix into the environment, forming gas channels and allowing pore water to be released (Fedorak et al., 2003; Guo et al., 2004). CO_2 produced during methane formation becomes dissolved in the pore water as HCO_3^- (bicarbonate) and decreases the pH of the alkaline pore water. This alters the chemistry of the pore water and allows cation exchange between the divalent cations on the surface of the clay particles, which in turn destabilizes the stable gel structure of the clays and increases densification (M. MacKinnon, personal communication). The exact mechanism of how microbial gas production increases densification is still under investigation.

4 Future Directions and Conclusions

4.1 Future directions

My thesis work has revealed unexpectedly high Archaeal and Bacterial diversity present in oil sands tailings. I did encounter some problems, which could easily be eliminated by changing the experimental methods of future studies. The use of MPN culture tubes, although it eliminated potential problems with interfering substances that may have been present in the tailings, may have caused a culture bias by preferentially enriching for species able to grow in the acetate-amended medium provided. I have shown that it is possible to easily extract genomic DNA from the oil sands MFT without the culture step, so future examination of tailings samples should be done without culturing. Also, using fresh tailings that were sampled aseptically and analyzed immediately without prolonged storage would eliminate doubt about the viability of the microbial communities, or introduction of contaminating microorganisms.

Comparing clone libraries constructed from acetate-amended and unamended tailings cultures, without the intermediate step of MPN culture would eliminate the culture bias that occurred in my experiments. This would clearly show how the methanogenic Archaeal and

Bacterial populations shift upon incubation with acetate.

I observed that many OTUs were often closely related to one another. This may have been due to microheterogeneity within the population rather than the true diversity, but also may have been a result of nucleotide misincorporation errors during the initial PCR amplification, because I did not use a proofreading polymerase, such as *Pfu*, in my PCR reactions. Although this may have created some artificial microheterogeneity in the clone libraries, it only resulted in my having to sequence more clones than I may have otherwise had to but did not significantly influence interpretation of the results. In future experiments, *Pfu* should be used in PCR amplification of community genomic DNA to reduce the possibility of nucleotide misincorporation.

Work should be done to confirm the different mechanisms of methane generation operating at different temperatures. [2-^{14}C]Acetate can be used to amend tailings microcosms, and the proportions of $^{14}CO_2$ and $^{14}CH_4$ compared to determine if acetoclastic methanogenesis or syntrophic acetate oxidation is the dominant pathway of methane generation. If acetoclastic methanogenesis is the dominant pathway, theoretically the methyl group of acetate will be converted to methane, and all of the radioactive label will be in the methane fraction. However, if the acetate is oxidized to CO_2 by the syntrophs, both carbons of acetate will end up as CO_2 (half ^{14}C-labelled). Approximately half of the CO_2 will be converted to $^{14}CH_4$ by the hydrogenotrophic methanogens and half will remain as $^{14}CO_2$. Therefore, the amount of radioactive label in the methane fraction *versus* the CO_2 fraction will indicate which the dominant process is. Inhibiting the acetoclastic methanogens with methyl fluoride (Roy et al., 1997) can also be used to determine if methane is being generated via hydrogenotrophic methanogenesis. Both of these experiments can be done at various temperatures to determine how methane generation changes with temperature. Constructing 16S rRNA gene clone libraries from the samples incubated at different temperatures would confirm that temperature causes changes in the microbial community structure, rather than just changes in activity of a constant community.

For future studies involving MFT taken from active settling basins, a less labor-intensive community fingerprinting technique should be used. Accumulation curves constructed from the Bacterial 16S rRNA clone libraries constructed from the neat tailings indicate that hundreds of clones need to be sampled to accurately represent the diversity present in the environment. This may not be necessary and requires a lot of time and money. Fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) or terminal restriction fragment length polymorphism (T-RFLP) allow changes between diverse communities to be easily monitored, and may be more applicable to the tailings environment, at least as initial screening method to demonstrate shifts in the communities.

[illegible]ble isotope probing (SIP) recently has been used to determine which microbial populations are active in an environment (Lueders et al., 2004). ^{13}C-Labeled substrate is added to an environmental sample and incubated for a certain period of time to allow the active microorganisms to metabolize the labeled substrate and incorporate the label into their DNA. The community DNA is then extracted, and the "heavy" DNA containing the ^{13}C-label is separated by centrifugation in a cesium chloride gradient. Clone libraries are constructed from the heavy DNA, and the clones present in the libraries should represent only the microorganisms which are active in the environment. This may decrease the number of irrelevant OTUs obtained in libraries constructed from the tailings, and decrease the time and effort required to determine "who is doing what" in this environment.

Although methanogenic species were not detected in the oil sands ores, there is evidence that they are present and actively producing methane in situ (M. MacKinnon, personal communication). It may be that the methanogens are localized in certain areas of the oil sand, and that the samples that I processed were from areas which did not have any of these microorganisms. It may be possible to find these microorganisms by using larger sample sizes. Large amounts of oil sand (e.g., 1 kg) could be slurried in pyrophosphate buffer, and the sand allowed to settle. Moderate heat could be used to separate the bitumen. The resulting suspension of fine tails can be filtered for use in bead-beating extractions to effectively increase the volume of ore being sampled for DNA. This may provide a better chance of finding the microorganisms present in the oil sands.

Oil sands samples could also be cultured using different substrates (acetate, formate, methanol, H_2+CO_2) and different conditions (temperature, pH), to grow any methanogenic microorganisms that are present in the oil sands. Clone libraries can then be constructed from the methanogenic microcosms to determine which species are living in the oil sands ore and are potentially the source of the methanogenic inoculum into MLSB. This method may introduce some culture bias; however, it may also allow any methanogens present in the oil sands in extremely low numbers to be detected.

4.2 Conclusions

In my thesis work, I wanted to determine the methanogenic microbial processes occurring in the tailings ponds of Syncrude Canada, Ltd. I used 16S rRNA gene clone libraries constructed from community DNA to attempt to determine the species of Bacteria and methanogenic Archaea present in the tailings, how the communities changed with addition of acetate, and if the same methanogens were present in the oil sands ore.

This study is the first attempt to determine the microbial species involved in methane production and densification of oil sands tailings. Previous microbiological studies have only confirmed the presence of various metabolic groups of microorganisms, but have not been able to identify the specific types of microorganisms, or the specific pathway of methane production. The novelty of this work presented many challenges, including the difficulty in working with slow-growing organisms in very contaminated samples. New methods needed to be developed to extract DNA from the hydrocarbon- and clay-contaminated tailings samples, and to work with a large number of clone libraries. The procedures developed and preliminary data presented here have laid the groundwork for future studies in this area.

I found that there was very little difference in the laboratory-incubated methanogenic populations in tailings samples either amended or unamended with acetate, but both sets of samples were dominated by hydrogenotrophic methanogens. The Bacterial populations were dominated by clostridia, a collection of anaerobic, endospore-forming fermentative bacteria. The Bacterial populations did not change dramatically with acetate amendment, with the exception of an increase in proportion of sequences related to the genus *Acetobacterium*. These sequences may represent homoacetogenic microorganisms which are participating in syntrophic acetate-oxidation in partnership with the hydrogenotrophic methanogens.

Dramatically different populations were seen when looking at the neat uncultured MFT. The methanogenic populations were instead dominated by acetoclastic methanogens, and the Bacterial populations were much more diverse. There was an almost complete dominance of the acetoclastic *Methanosaeta* spp., with almost no sequences related to hydrogenotrophic methanogens. Sequences related to SRB and syntrophic microorganisms as well as other members of the α-, β-, γ-, and δ-proteobacteria were detected in the libraries constructed from the neat MFT. The detection of these species supports the proposed scheme of methanogenic degradation of hydrocarbons in the tailings.

I was not able to amplify Archaeal 16S rRNA sequences from the oil sands ore, but was able to amplify Bacterial 16S rRNA sequences. Further work in this area may eventually detect methanogens in the oil sands ore.

This research has been valuable because it provided the initial look at the identities of the microbes in communities generating methane in MLSB and WIP, and will support further research to describe and define methanogenesis *in situ*. This may eventually aid the design of strategies to increase MFT densification and eventual use in reclamation. I have also generated a large electronic database of ARDRA patterns and the species represented by the OTUs, which will greatly decrease the effort required for analysis of 16S rRNA gene clone libraries constructed from the tailings in the future.

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6 Appendices

Appendix 1: Archaeal HaeIII restriction pattern fragment sizes

Pattern Name	Fragment Size (bp)	Total Size
AC	183, 107, 93, 66	449
AG	245, 179, 117, 110, 83	734
AJ	277, 268, 121, 48	714
N	255, 179, 110, 90, 62	696
AN	277, 259, 186, 121, 59	902
AL	286, 250, 121, 45	702
AK	286, 273, 179, 62	800
AF	291, 241, 179, 55	766
AB	300, 250, 183, 159, 55	947
AM	295, 183, 45	523
W	309, 200, 148, 79, 45	781
T	300, 210, 220	730
U	311, 240, 100, 17	668
AI	270, 220, 161, 30	1035
F	300, 225, 91, 13	681
AH	300, 240, 91, 22	629
Q	340, 300, 280, 60	653
X	342, 305, 285, 52	980
V	358, 126, 35	984
C	363, 245, 43	519
O	363, 342, 135	651
Z	419, 230, 170, 61	840
I	413, 245, 122	880
P	425, 280, 205, 70	780
A	433, 216, 166, 107, 41	980
AO	422, 296, 131, 38	963
H	439, 216, 128, 24	887
L	444, 284, 114, 24	807
J	439, 330, 117, 28,	866
D	444, 114, 41	914
Y	461, 216, 166, 52	599
S	513, 478	895
R	519, 483	991
G	525, 268, 110, 38	1002
AA	657, 176, 93, 66	941
K	636, 348, 121, 45	992
E	636, 216, 128, 55	1150
B	629, 268, 107	1004
M	708, 280	988

Appendix 2: Archaeal CfoI restriction pattern fragment sizes

Pattern Name	Fragment sizes (bp)	Total Size
AB	272, 260, 236, 111, 64	943
X	314, 300, 276, 175, 46	1111
K	345, 314, 132, 50	841
AF	350, 305, 132, 46	833
Y	355, 305, 280, 157, 50	1147
W	364, 336, 288, 136, 54	1178
AG	364, 276, 164	804
P	377, 305, 146, 71	899
D	382, 323, 71	776
AE	411, 309, 268, 161	1149
G	444, 284	728
T	456, 345, 260	1061
V	425, 252, 46	723
AD	450, 257, 177	884
AC	431, 252, 213	896
U	425, 243, 54	722
Z	438, 257, 177	872
H	456, 316, 261, 115, 54	1202
AA	475, 353	828
L	488, 431, 226, 65	1210
S	470, 440, 190	1100
C	444, 265, 65	774
A	488, 456, 235, 65	1244
E	456, 248, 62	766
M	553, 448, 137	1138
O	600, 424, 103, 43	1170
R	600, 414, 173, 37	1224
Q	663, 433, 204, 50	1350
J	688, 391, 43	1122
B	700, 476, 53	1229
I	720, 313, 150, 50	1233
F	727, 438, 43	1208
N	983, 173	1156

Appendix 3: List of Archaeal OTUs, indicating HaeIII and CfoI pattern, representative clone sequenced and closet match in GenBank.

OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity
Euryarchaeota					
Methanomicrobiales					
Methanocorpusculum					
A1	A	E	#1 - 6E	*Methanocorpusculum labreanum* strain DSM 4855; AY260436	97
A2	C	L	#17 - 1F	*Methanocorpusculum parvum* strain DSM 3829; AY260435	99
A3	L	B	#17 - 9D	*Methanocorpusculum parvum* strain DSM 3829; AY260435	99
A4	P	F	#17 - 4B	*Methanocorpusculum parvum* strain DSM 3829; AY260435	99
A5	V	B	#13 - 10B	*Methanocorpusculum parvum* strain DSM 3829; AY260435	99
A6	W	D	#17 - 7A	*Methanocorpusculum parvum* strain DSM 3829; AY260435	99
A7	A	H	#1 - 5C	*Methanocorpusculum parvum* strain DSM 3829; AY260435	95
A8	A	L	#10 - 5A	*Methanocorpusculum parvum* strain DSM 3829; AY260435	98
A9	E	G	#1 - 6B	*Methanocorpusculum parvum* strain DSM 3829; AY260435	97
A10	H	C	#1 - 11B	*Methanocorpusculum parvum* strain DSM 3829; AY260435	97
A11	A	A	#1 - 1C	*Methanocorpusculum parvum* strain DSM 3829; AY260435	96
A12	A	C	#1 - 1H	*Methanocorpusculum parvum* strain DSM 3829; AY260435	96
Methanocalculus					
A13	A	B	#1 - 6D	*Methanocalculus pumilus;* AB008853	97
A14	AA	F	#20 - 1E	*Methanocalculus pumilus;* AB008853	98
A15	B	B	#1 - 1B	*Methanocalculus pumilus;* AB008853	96
	B	B	#8 - 2D	*Methanocalculus pumilus;* AB008853	98
	B	B	#8 - 5B	*Methanocalculus pumilus;* AB008853	97
A16	B	F	#1 - 1G	*Methanocalculus pumilus;* AB008853	97
	B	F	#8 - 1H	*Methanocalculus pumilus;* AB008853	98
A17	B	I	#9 - 6H	*Methanocalculus pumilus;* AB008853	97
A18	B	L	#8 - 6C	*Methanocalculus pumilus;* AB008853	97
A19	B	M	#9 - 4G	*Methanocalculus pumilus;* AB008853	97
A20	B	V	#19 - 8G	*Methanocalculus pumilus;* AB008853	98
A21	E	B	#20 - 8D	*Methanocalculus pumilus;* AB008853	98
A22	G	B	#9 - 3F	*Methanocalculus pumilus;* AB008853	96
A23	G	E	#9 - 4A	*Methanocalculus pumilus;* AB008853	98
A24	G	F	#1 - 8H	*Methanocalculus pumilus;* AB008853	97
A25	K	F	#1 - 1F	*Methanocalculus pumilus;* AB008853	97
A26	M	B	#8 - 10E	*Methanocalculus pumilus;* AB008853	97
A27	Q	B	#9 - 6D	*Methanocalculus pumilus;* AB008853	97
A28	Q	E	#9 - 8C	*Methanocalculus pumilus;* AB008853	97
A29	U	I	#13 - 2H	*Methanocalculus pumilus;* AB008853	98
A30	X	F	#18 - 4E	*Methanocalculus pumilus;* AB008853	98
A31	Y	E	#19 - 5D	*Methanocalculus pumilus;* AB008853	98
A32	Z	E	#19 - 7F	*Methanocalculus pumilus;* AB008853	98
A33	A	F	#1 - 6A	*Methanocalculus taiwanese* strain P2F9704a; AF172443	97
A34	B	E	#1 - 4F	*Methanocalculus taiwanese* strain P2F9704a; AF172443	98
A35	B	O	#9 - 10E	*Methanocalculus taiwanese* strain P2F9704a; AF172443	97
A36	B	Q	#10 - 4F	*Methanocalculus taiwanese* strain P2F9704a; AF172443	98
A37	B	S	#13 - 4C	*Methanocalculus taiwanese* strain P2F9704a; AF172443	98
A38	E	F	#1 - 4C	*Methanocalculus taiwanese* strain P2F9704a; AF172443	98
A39	F	F	#8 - 8D	*Methanocalculus taiwanese* strain P2F9704a; AF172443	96
A40	P	B	#9 - 3G	*Methanocalculus taiwanese* strain P2F9704a; AF172443	98
Methanoculleus					
A41	E	D	#1 - 1E	*Methanoculleus chikugoensis;* AB03795	96
A42	D	W	MLSB 6m 12C	*Methanoculleus palmaeoli;* Y16382 Uncultured archaeon clone PL-9A5; AY570665	96 98
A43	D	AF	WIP 6m 6F	*Methanoculleus palmaeoli;* Y16382 Uncultured archaeon clone PL-9A5; AY570665	97 99
A44	A	D	#1 - 11E	*Methanoculleus palmeoli*; Y16382	97
A45	B	D	#1 - 6C	*Methanoculleus palmeoli*; Y16382	97
A46	D	D	#1 - 8A	*Methanoculleus palmeoli*; Y16382	96
A47	D	K	#8 - 6B	*Methanoculleus palmeoli*; Y16382	97

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity
Methanosarcinales					
Methanosarcina					
A48	C	E	#1 - 2F	*Methanosarcina acetivorans;* M59137	98
A49	C	U	#19 - 11B	*Methanosarcina lacustris* strain MS; AY260431	98
A50	C	F	#1 - 7B	*Methanosarcina mazei* strain Goe1; AE013440	97
A51	C	T	#13 - 7A	*Methanosarcina mazei* strain Goe1; AE013440	99
A52	C	V	#19 - 6C	*Methanosarcina mazei* strain Goe1; AE013440	99
A53	C	A	#1 - 4A	*Methanosarcina mazei* strain MT; AY260432	98
A54	C	G	#1 - 5B	*Methanosarcina mazei* strain MT; AY260432	96
A55	I	G	#1 - 11C	*Methanosarcina mazei* strain MT; AY260432	98
A56	L	E	#19 - 4H	*Methanosarcina mazei* strain SarPi; AF028691	99
Methanomethylovorans					
A57	J	B	#8 - 1G	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
A58	J	F	#1 - 12D	*Methanomethylovorans hollandica* strain ZB (AF120163)	99
	J	F	#1 - 12D	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
	J	F	#10 - 8F	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
A59	J	M	#8 - 10C	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
A60	L	J	#8 - 1A	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
A61	O	B	#8 - 12A	*Methanomethylovorans hollandica* strain ZB (AY260433)	98
A62	Y	U	#19 - 3B	*Methanomethylovorans hollandica* strain ZB (AY260433)	99
Methanosaeta					
A63	AG	I	WIP 6m 8C	*Methanosaeta concilii* (X51423)	99
A64	N	W	WIP 6m 9C	*Methanosaeta concilii* (X51423)	99
A65	N	I	#8 - 3F	Uncultured *Methanosaeta* sp. clone KuA1 (AB077211)	98
	N	I	MLSB 6m 7A	Uncultured *Methanosaeta* sp. clone KB-1 2 (AY780569)	98
A66	AF	I	MLSB 10m 2B	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A67	AH	I	MLSB 10m 6H	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A68	AJ	I	MLSB 10m 12E	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A69	AL	Ia	MLSB 20m 11D	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A70	F	I	#1 - 7C	*Methanosaeta* sp. Clone A1 (AJ133791)	99
	F	I	MLSB 6m 1A	*Methanosaeta* sp. Clone A1 (AJ133791)	99
	F	I	MLSB 6m 2B	*Methanosaeta* sp. Clone A1 (AJ133791)	98
	F	I	MLSB 6m 2C	*Methanosaeta* sp. Clone A1 (AJ133791)	95
A71	F	Ia	MLSB 6m 1B	*Methanosaeta* sp. Clone A1 (AJ133791)	98
	F	Ia	MLSB 6m 1C	*Methanosaeta* sp. Clone A1 (AJ133791)	99
	F	Ia	MLSB 6m 1D	*Methanosaeta* sp. Clone A1 (AJ133791)	97
A72	F	K	MLSB 10m 2E	*Methanosaeta* sp. Clone A1 (AJ133791)	98
A73	F	P	MLSB 30m 9C	*Methanosaeta* sp. Clone A1 (AJ133791)	98
A74	F	X	WIP 6m 2D	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A75	L	Ia	WIP 6m 4E	*Methanosaeta* sp. Clone A1 (AJ133791)	98
A76	O	I	WIP 20m 10A	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A77	O	Ia	MLSB 30m 12E	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A78	P	E	#18 - 2B	*Methanosaeta* sp. Clone A1 (AJ133791)	99
A79	N	Ia	MLSB 6m 7B	Uncultured archaeon clone PL-35A3 (AY570656) Uncultured *Methanosaeta* sp. Clone KuA1 (AB077211)	99 98
A80	AG	Ia	MLSB 10m 2H	Uncultured *Methanosaeta* sp. clone KB-1 2 (AY780569)	95
Methanolobus					
A81	C	I	#8 - 7C	*Methanolobus taylorii* (U20154)	95
Methanobacteriales					
Methanobacterium					
A82	A	U	#19 - 7E	*Methanobacterium formicicum* strain FCam (AF028689)	99
Methanobrevibacter					
A83	T	P	#10 - 3E	*Methanobrevibacter arboriphilus* (AB065294)	93

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity
Crenarchaeota					
Unclassified Crenarchaeota					
A92	L	Y	MLSB 20m 12D	Crenarchaeotal sp. clone pJP (L25301)	92
A93	AO	AF	WIP 20m 6B	Crenarchaeotal sp. clone pJP (L25301)	93
A94	AO	Y	WIP 20m 2E	Crenarchaeotal sp. clone pJP (L25301)	92
A95	J	W	WIP 20m 10H	Crenarchaeotal sp. clone pJP (L25301)	92
A96	J	X	MLSB 20m 4B	Crenarchaeotal sp. clone pJP (L25301)	92
A97	L	D	MLSB 30m 1H	Crenarchaeotal sp. clone pJP (L25301)	92
A98	L	X	MLSB 30m 5F	Crenarchaeotal sp. clone pJP (L25301)	92
A99	N	X	WIP 10m 5F	Crenarchaeotal sp. clone pJP (L25301)	92
A100	AO	W	WIP 20m 6G	Uncultured crenarchaeote clone OPPD014 (AY861950)	94
A101	F	W	MLSB 30m 2E	Uncultured archaeon 16S rRNA, clone:OHKA4.59 (AB094541)	97
A102	AK	AF	WIP 10m 9G	Uncultured archaeon clone:ASC40 (AB161339)	96
A103	AK	AA	WIP 6m 2C	Unidentified archaeon 16S rRNA gene, clone 122 (AJ831142)	98
A104	AM	P	WIP 20m 8F	Unidentified archaeon 16S rRNA gene, clone 122 (AJ831142)	99
A105	AM	W	WIP 20m 1F	Unidentified archaeon clone vadinDC69 (U81774)	97
A106	AK	W	MLSB 30m 6G	uncultured archaeon 16S rRNA, clone ss016b (AJ969773)	96
A107	AN	Z	WIP 6m 9B	uncultured archaeon 16S rRNA gene, clone ss043 (AJ969795)	93
A108	N	AC	WIP 6m 3C	uncultured archaeon 16S rRNA gene, clone ss043 (AJ969795)	93
A109	N	Z	MLSB 30m 7F	uncultured archaeon 16S rRNA gene, clone ss043 (AJ969795)	92
Chimeras					
A84	AB	AE	WIP 6m 4H	Uncultured euryarchaeote clone KuA13 (AB077223)	99
A85	AB	I	MLSB 6m 3B	Uncultured euryarchaeote clone KuA13 (AB077223)	98
A86	AB	Ia	MLSB 6m 1F	Uncultured euryarchaeote clone KuA13 (AB077223)	99
A87	AC	AG	WIP 20m 7A	Uncultured euryarchaeote clone OPPD017 (AY861953)	96
A88	AF	Ia	MLSB 6m 2H	Uncultured archaeon 44A-1 (AF424765)	98
A89	AI	Ia	MLSB 10m 3E	Uncultured euryarchaeote clone KuA13 (AB077223)	99
A90	AK	Ia	MLSB 20m 1D	Uncultured euryarchaeote clone KuA13 (AB077223)	99
A91	AM	Ia	MLSB 30m 5D	Uncultured euryarchaeote clone KuA13 (AB077223)	99
Unsequenced clones					
A110	AK	I	WIP 10m 7F		
A111	AL	I	WIP 10m 9H		

Appendix 4: Bacterial HaeIII restriction pattern fragment sizes

Pattern Name	Fragment Size	Total Size
BE	261, 147, 97	505
AK	268, 250, 147, 97, 50	812
AM	270, 230, 170, 60	730
AU	271, 236, 175, 75, 25	782
BM	300, 246, 178, 138, 84, 28	974
AZ	296, 239, 141, 56	735
AE	309, 257, 221, 150	946
Ya	304, 264, 184, 125, 84	977
BJ	309, 147	456
Y	309, 200, 169	691
AS	326, 243, 153, 88	816
AV	304, 147	467
AO	321, 290, 210, 135, 81	1060
AW	646, 252, 127	1048
BO	295, 185, 146	626
BPA	368, 267, 219, 158, 85	1112
AQ	374, 290, 131	814
AL	395, 248, 210, 154, 65	1072
BP	406, 358, 295, 188, 88	1335
AH	337, 276, 238, 169, 112	1132
BK	475, 358, 276, 200, 69	1378
BB	425, 290, 185, 65	965
BCA	488, 332, 243, 200, 54	1317
U	425, 281, 173, 69	948
BH	457, 309, 148, 73, 27	1014
BCA	476, 313, 236, 115, 33	1173
BDB	419, 348, 322, 250, 73	1439
MC	476, 275, 173, 85, 36	1045
MA	457, 239, 204, 176, 145	1269
BL	471, 218, 148, 109, 67	1031
R	452, 313, 282, 176, 91	1344
M	462, 282, 191, 55	990
D	486, 282, 94, 21	883
MB	452, 282, 214, 145	1108
BDA	476, 335, 304, 221, 185	1521
AY	448, 326, 173, 91	1038
BD	482, 348, 324, 233, 155	1576
AJB	488, 186, 141	849
BG	488, 283, 148	943
AT	488, 324, 242, 148	1230
E	500, 465, 242, 69	1276
B	494, 465, 283, 59	1301
C	488, 471, 296, 100, 62	1417
AD	494, 329, 114	965
AJA	488, 221, 152	902
MD	488, 292, 217, 145, 59	1201
ATB	471, 305, 250, 172	1226
ATA	482, 279, 197, 148	1123
AN	490, 330, 270, 200	1290
P	490, 460, 300, 150, 60	1460
BT	500, 420, 300, 50	1270
BTA	500, 420, 300, 210, 50	1480
BQ	520, 280, 230, 180, 90	1300
BF	520, 330, 210, 150, 60	1270
X	520, 450, 250	1220
AJ	530, 230, 210, 150, 140	1260
AF	530, 290, 180, 120	1120
V	540, 450, 340	1330
Q	550, 350, 280, 170, 130	1480
L	570, 440, 280, 50	1340
BU	577, 460, 241, 154	1470
AP	577, 282, 165, 77	1101
APA	600, 291, 162, 65	1118
BN	608, 311, 259, 169, 54	1401

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Pattern Name	Fragment Size (bp)	Total Size
BI	683, 291, 245, 162, 58	1481
AR	592, 344, 218	1192
BW	600, 394, 214, 138, 54	1400
I	650, 273, 173, 65	1161
BS	650, 300, 273, 165	1426
K	667, 223, 112	1021
AC	667, 328, 181, 88	1279
AX	658, 389, 241, 62	1350
BR	693, 258, 208	1159
AGA	809, 336, 269	1444
AI	800, 345, 250, 90	1485
AG	790, 330, 260, 190	1570
O	800, 260, 170, 70	1300
AB	809, 359, 197,	1365
H	836, 254, 137, 87	1314
BV	864, 332,	1243
F	855, 163, 50	1068
T	920, 265, 50,	1235
N	910, 332, 163, 100	1525
J	960, 254, 163,	1397
Z	943, 340, 148	1431
S	1000, 264,	1264
A	1103, 327	1430
W	1138, 200, 144	1482
G	1241, 353,	1594
AA	1431, 63	1494

Appendix 5: Bacterial CfoI restriction pattern fragment sizes

Pattern Name	Fragment Size (bp)	Total Size
BH	325, 304, 207, 69	905
BV	371, 215, 149, 71	806
BK	367, 270, 204, 151	992
BF	363, 338, 256, 197, 143, 63	1360
AP	367, 293, 222, 151, 66	1099
BKA	342, 281, 183, 146, 69	1021
BT	410, 338, 285, 215, 174, 63	1499
BM	440, 270, 230, 194, 154, 120	1408
BU	500, 470, 310, 200, 160	1640
ANA	450, 333, 285, 166, 51	1285
AEA	544, 346, 244, 177, 109, 43	1463
T	465, 371	836
F	472, 373, 230, 145, 61	1281
AY	521, 461, 204, 52	1238
Q	483, 336, 190, 58	1067
AU	467, 373, 235, 181, 142, 65	1463
BW	478, 382, 274, 194, 132, 74	1534
BC	478, 265, 190, 61	994
AC	543, 364, 300, 181, 97	1485
AWA	489, 350, 194, 77	1110
AL	514, 332, 200, 77	1123
BO	510, 240, 200, 150, 30	1130
AR	510, 280, 210	1000
AE	521, 300, 213, 165	1199
AK	547, 365, 162, 52	1126
AUA	520, 370, 282, 62	1234
AB	627, 547, 479, 205, 55	1913
N	547, 468, 268, 200	1483
BOB	507, 370, 282, 131, 48	1338
W	533, 463, 400, 259, 52	1707
G	520, 479, 250, 200, 38	1487
O	513, 317, 38	868
U	489, 348, 190, 38	1065
AXB	520, 479, 264, 190, 152, 31	1636
ALA	520, 474, 348, 176, 31	1549
BOA	507, 259, 227, 200, 31	1224
AQ	556, 359, 221, 171, 84	1391
BWA	519, 355, 289, 187, 79	1429
AA	513, 182, 82	777
BI	530, 480, 220, 200, 140	1570
BX	538, 500, 411, 263, 163, 68	1943
AQB	544, 359, 242, 168, 118, 79	1510
AWB	531, 368, 300, 124, 66	1389
AD	525, 258, 187, 116, 63	1149
BY	525, 171, 61	757
AZ	525, 489, 253, 179, 53	1499
AN	500, 350, 289, 161, 53	1353
AW	540, 520, 430, 290	1780
AQA	563, 352, 250, 185	1350
BWB	537, 348, 247, 179, 103, 47	1461
CB	526, 336, 118, 44	1024
BN	574, 438, 300, 159, 129, 41	1641
X	579, 490, 344, 247	1660
AO	650, 481, 405, 124, 44	1704
BQA	625, 396, 312, 162, 138, 47	1680
AF	631, 467, 247, 44	1389
AHA	694, 638, 336, 44	1712
BQ	644, 352, 297, 159	1452
AI	650, 516, 316, 207, 47	1736
BR	669, 340, 191, 165, 56	1421
P	673, 512, 327, 196, 50	1758
H	680, 524, 327, 200, 50	1781
AH	700, 541, 364, 143, 54	1802
L	700, 547, 46	293

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Continued from previous page

Pattern Name	Fragment Size (bp)	Total Size
AJ	653, 489, 421, 121, 43	1727
AHB	690, 550, 370, 150	1760
BS	693, 633, 336, 43	1705
BZ	700, 510, 290	1500
AG	693, 261, 225	1179
V	660, 512, 474	1646
BEA	723, 350, 293, 154, 32	1552
BEA	731, 355, 332, 146, 43	1607
M	692, 507, 488, 56	1743
BG	700, 513, 357, 59	1629
BB	736, 63	799
BP	727, 74	801
BD	718, 471, 227, 70	1486
AT	755, 727, 488, 277, 205, 93	2545
BL	755, 527, 193, 159, 104	1738
AM	800, 533, 429, 96	1858
AV	718, 507, 352, 93	1670
Ra	911, 400, 352, 286, 111	2060
I	745, 357, 167, 89	1358
K	873, 482, 400, 348, 291, 178	2691
J	830, 490, 130	1450
A	831, 490, 342, 65	1728
S	838, 490, 204, 65	1597
B	823, 511, 321, 62	1717
BA	869, 358, 256, 68	1551
AS	885, 471, 256, 181, 62	1855
C	854, 522, 256, 65.	1697
R	1022, 230, 162, 76, 54	1544
Y	980, 520, 220	1720
BJ	1233, 259, 62	1554
Z	1089, 500, 57	1646
D	1311, 1211, 638, 517, 65	3742
Ca	1244, 358, 143, 59	1804
E	1222, 490, 62	1774

Appendix 6: List of Bacterial OTUs, indicating HaeIII and CfoI pattern, representative clone sequenced and closest match in GenBank and RDP II. Similarity values are based on partial sequences (~500 bp) unless otherwise indicated.

OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity GenBank	% Identity RDP
Proteobacteria						
Alphaproteobacteria						
B1*	AP	Bn	MLSB 30m 3F	*Caulobacter* sp.; DSM 10556; AJ227788	99	0.972
B2	BP	At	WIP 6m 6F	magnetite-containing magnetic vibrio; MV1; L06455	94	0.711
				Hyphomicrobium zavarzinii (T); ZV-622; Y14305	–	0.589
B3*	Ma	Bm	WIP 6m 7D	*Sphingomonadaceae* bacterium MWH-CaK2; AJ565420	94	0.741
B4	AY	I	WIP 10m 7E	uncultured bacterium; B3NR56D5; AY957908	98	0.987
				Uncultured *Azospirillum* sp. clone GCPF16; AY129790	97	–
Betaproteobacteria						
B5	Atb	AL	WIP 10m 1B	beta proteobacterium PB7; AY686732	98	0.971
B6	AT	Ae	MLSB 10m 6B	uncultured beta proteobacterium; B-Y34; AY622248	98	0.903
				Rhodoferax ferrireducens (T); T118; AF435948	97	0.882
B7*	AT	AL	MLSB 10m 6D	uncultured beta proteobacterium; B-Y34; AY622248	97	0.915
				Rhodoferax ferrireducens (T); T118; AF435948	98	0.919
B8	BD	Ae	MLSB 20m 8G	uncultured beta proteobacterium; B-Y34; AY622248	97	0.896
				Rhodoferax ferrireducens (T); T118; AF435948	97	0.887
B9	AT	AA	WIP 10m 6C	uncultured beta proteobacterium; B-Y34; AY622248	97	0.899
				Rhodoferax ferrireducens (T); T118; AF435948	96	0.880
B10	ATa	AL	WIP 10m 8B	uncultured bacterium; RB13C10; AF407413	97	0.947
				Rhodoferax antarcticus; Fryx1; AY609198	96	0.925
B11	Bda	Ae	WIP 6m 2C	uncultured bacterium; RB9C1; AF407392	98	0.973
				Rhodoferax antarcticus; Fryx1; AY609198	97	0.933
B12	AS	Ae	MLSB 10m 5G	*Acidovorax defluvii* (T); BSB411; Y18616	99	0.963
B13	AS	Bi	MLSB 20m 1C	*Acidovorax defluvii* (T); BSB411; Y18616	98	0.924
				denitrifying Fe<II>-oxidizing bacteria; BrG1; U51101	99	0.937
B14	AS	AL	WIP 10m 3G	*Acidovorax facilis*; DSM 649; AJ420324	98	0.902
B15	AV	AL	MLSB 10m 7F	*Malikia granulosa*; type strain: P1; AJ627188	98	0.900
				Hydrogenophaga taeniospiralis (T); ATCC 49743; AF078768	98	0.873
B16	AS	Ar	MLSB 20m 7C	*Hydrogenophaga taeniospiralis* (T); ATCC 49743; AF078768	99	0.947
B17	AR	Aha	MLSB 10m 7E	uncultured beta proteobacterium; AKYG1037; AY921769	97	0.882
B18	BC	Ae	MLSB 20m 2F	beta proteobacterium CDB21; AB194096	95	0.850
				Sterolibacterium denitrificans (T); Chol-1S; AJ306683	–	0.729
B19	AI	AN	MLSB 6m 2G	uncultured beta proteobacterium; ccs265; AY133064	96	0.814
				Propionivibrio limicola (T); GolChi1 T; AJ307983	–	0.705
B20	AI	Ae	WIP 20m 10D	uncultured beta proteobacterium; ccs265; AY133064	95	0.812
				Thiobacillus aquaesulis; U58019	–	0.738

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity GenBank	% Identity RDP
B21	Al	Ah	MLSB 10m 1F	Uncultured soil bacterium clone L1A.6E11; AY988982	98	–
				Thiobacillus denitrificans (T); NCIMB 9548; AJ243144	95	0.790
B22	AG	Ah	MLSB 10m 2E	beta proteobacterium 5Z-C1; AJ224618	98	0.928
				Thiobacillus denitrificans (T); NCIMB 9548; AJ243144	96	–
	AG	Ah	MLSB 30m 1D	beta proteobacterium TBW3; AJ224617	98	0.928
				Thiobacillus denitrificans (T); NCIMB 9548; AJ243144	96	0.844
B23	AG	Aw	MLSB 10m 7D	uncultured bacterium; RB7C6; AF407385	99	0.949
				Thiobacillus denitrificans (T); NCIMB 9548; AJ243144	94	0.894
B24*	Aga	Ah	MLSB 20m 2G	uncultured bacterium isolate cMM319-15; AJ536816	97	0.924
				Thiobacillus denitrificans strain NCIMB; AJ243144	96	0.882
B25	Aga	Aea	MLSB 20m 6A	uncultured bacterium; RB7C6; AF407385	99	1.000
				uncultured *Thiobacillus* sp. KF-Gitt2-40; AJ295643	98	0.930
B26	AG	Bi	MLSB 30m 6A	uncultured bacterium; RB7C6; AF407385	99	0.972
				uncultured *Thiobacillus* sp. KF-Gitt2-40; AJ295643	97	0.880
B27	AG	Aea	WIP 20m 3A	uncultured bacterium; RB7C6; AF407385	99	0.950
B28	AG	Ahb	WIP 6m 2A	uncultured beta proteobacterium; ccspost271; AY133108	98	0.948
				Thiobacillus denitrificans strain NCIMB; AJ243144	95	–
B30	BK	Aw	MLSB 30m 1B	uncultured beta proteobacterium; 36-9; AF351219	96	0.925
B31	Bca	Ak	MLSB 6m 1F	uncultured beta proteobacterium; 36-9; AF351219	97	0.862
B32	AT	Aw	WIP 20m 1G	uncultured beta proteobacterium; 36-9; AF351219	98	0.886
B33	AS	Ah	MLSB 30m 2E	beta proteobacterium Rufe9; AY235687	92	–
				Denitratisoma oestradiolicum; AcBE2-1; AY879297	–	0.741
B34	Al	Aw	MLSB 10m 14H	beta proteobacterium MWH-UniP1; AJ565421	97	0.897
				Burkholderia cepacia (T); ATCC 25416T; U96927	–	0.711
B35	AS	Aq	WIP 20m 5D	uncultured bacterium; UTFS-OF08-18; AB166784	94	0.816
				Dechloromonas sp. NM; AF170355	94	0.800
B36	BJ	Aw	WIP 6m 8A	uncultured bacterium; 35-45D; AY955092	97	0.904
				Thauera phenylacetica (T); B4P = DSM 14743*; AJ315678	97	0.876
B37	Ya	Ah	WIP 10m 11C	*Azoarcus* sp.; M3; Y11041	–	0.696
				uncultured *Methylophilaceae* bacterium; 10-3Ba28; AY360550	–	0.700
Gammaproteobacteria						
B38	AJ	I	MLSB 10m 17H	uncultured bacterium; 5H_90; AY546512	97	0.892
				Dokdonella koreensis; DS-123; AY987368	–	0.737
B39	AJ	Be	MLSB 10m 2A	uncultured bacterium; 5H_90; AY546512	–	0.908
				Lysobacter gummosus; KCTC 12132; AB161361	–	0.722
B40	AJ	Bea	MLSB 10m 3B	uncultured bacterium; 5H_90; AY546512	97	0.906
				Xanthomonas campestris; XCC15; AF123092	–	0.716
B41	Y	AL	MLSB 20m 2E	Uncultured Gammaproteobacteria clone 4P36; AJ871058	99	–
				Dokdonella koreensis; DS-123; AY987368	92	0.733

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity GenBank	RDP
B42	AH	Aqa	MLSB 10m 1D	Uncultured eubacterium clone IAFR510; AF270959	96	–
				Thialkalivibrio thiocyanodenitrificans strain ARhD; AY360060	88	–
B43	AJa	Aq	MLSB 10m 8D	Uncultured eubacterium clone IAFR510; AF270959	95	–
				Methylocaldum sp. E10a; AJ868426	90	0.586
B44	AJ	Aea	MLSB 20m 11C	Uncultured eubacterium clone IAFR510; AF270959	95	–
				Codakia costata gill symbiont; L25712	–	0.607
B45	AH	Aqb	WIP 6m 4D	endosymbiont of *Seepiophila jonesi*; AY129105	–	0.598
				Uncultured soil bacterium clone PYR10d11; DQ123671	95	–
B46	AH	Aq	MLSB 10m 16G	Uncultured eubacterium clone IAFR510; AF270959	97	–
				Thialkalivibrio thiocyanodenitrificans strain ARhD; AY360060	89	–
B47	AH	Bf	MLSB 20m 11D	Uncultured eubacterium clone IAFR510; AF270959	100	–
B48	AH	Bk	MLSB 20m 3B	Uncultured bacterium clone CCSD_DF1080_B22; AY820699	94	–
				Methylocaldum sp. E10a; AJ868426	–	–
B49	AH	Bka	WIP 10m 12C	Uncultured eubacterium clone IAFR510; AF270959	98	–
				Halomonas sp. A-9; AY914060	–	0.608
B50	AJ	Aqb	WIP 20m 3H	Codakia costata gill symbiont; L25712	–	0.620
				Thialkalivibrio thiocyanodenitrificans strain ARhD; AY360060	90	–
B51	Aja	Aqa	MLSB 10m 10A	Uncultured gamma proteobacterium clone LTUG09056; AY144261	91	–
				Methylocaldum szegediense (T); OR2; U89300	96	–
B52	AJ	Bf	MLSB 20m 2C	uncultured gamma proteobacterium; B-AC40; AY622251	99	0.958
				Methylosarcina fibrata (T); AML-C10; AF177296	96	0.697
B53*	AJ	Bk	MLSB 30m 5A	uncultured gamma proteobacterium; B-AC40; AY622251	98	0.953
B54	AJ	Bz	MLSB 30m 6B	uncultured gamma proteobacterium; B-AC40; AY622251	95	0.946
				phototrophic bacterium (T); DSM 2111; X93478	–	0.710
B55	Mb	Aua	WIP 6m 10G	uncultured bacterium; TSBX24; AB186843	93	0.725
				Microbulbifer salipaludis (T); SM-1; AF479688	–	0.657
B56*	AM	O	MLSB 10m 2G	uncultured bacterium; 69-7G; AY955095	99	0.937
				Pseudomonas alcaligenes; LB19; AF390747	97	0.860
B57*	I	Bob	WIP 10m 9B	Pseudomonas sp. DVS6dla ; AY864636	99	0.972
Deltaproteobacteria						
B58	AV	AA	MLSB 10m 11D	uncultured bacterium; KNA6-EB15; AB179691	94	0.845
				Desulfobulbus sp. BG25; U85473	97	–
B59	Mb	Ae	MLSB 30m 3C	uncultured bacterium; KNA6-EB15; AB179691	95	0.811
				Syntrophomonas wolinii 16S rRNA gene; X7090	87	–
B60	Mc	Bk	WIP 10m 4D	uncultured bacterium; 1013-1-CG26; AY532547	97	0.885
				Methylocaldum sp. E10a; AJ868426	88	–
B61	Ya	AA	WIP 10m 8C	uncultured bacterium; KNA6-EB15; AB179691	94	0.761
B62	BJ	Bk	WIP 20m 10H	uncultured proteobacterium; ccs202; AY133065	97	0.904

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity GenBank	% Identity RDP
B63	Ya	Bo	WIP 6m 4F	uncultured bacterium; KNA6-EB15; AB179691	95	0.768
				Desulfobulbus sp. BG25; U85473	89	–
B64	ATa	Ae	WIP 10m 8E	uncultured delta proteobacterium; ML320J-28; AF458285	89	0.700
				Desulfocapsa thiozymogenes (T); DSM 7269; X95181	–	0.512
B65*	AE	Bw	WIP 20m 1H	uncultured bacterium; sipK108; AJ307944	97	0.834
				Desulfocapsa sp. Cad626; AJ511275	95	0.803
B66	Ax	Al	MLSB 6m 10E	uncultured bacterium; SHA-42; AJ306771	92	0.693
B67	BG	At	WIP 6m 5F	uncultured delta proteobacterium 36-11; AF351220	97	0.833
B68	Ya	At	WIP 6m 7F	uncultured bacterium; FW99; AF523966	93	0.732
				uncultured *Syntrophus* sp.; PD-UASB-35; AY261813	92	0.679
B69	Ya	Bea	WIP 6m 8G	uncultured bacterium; FW99; AF523966	94	0.726
				uncultured *Syntrophus* sp.; PD-UASB-40; AY261815	91	0.656
B70*	M	Bb	MLSB 6m 11C	uncultured eubacterium WCHB1-12; AF050534	99	0.971
				Syntrophus sp. 16S rRNA gene, partial, Clone B1; AJ133794	95	0.808
B72	AJ	At	MLSB 10m 14E	uncultured delta proteobacterium; 44a-B1-10; AY082457	93	0.693
			MLSB 10m 14G	uncultured delta proteobacterium; 44a-B1-10; AY082457	92	0.685
				uncultured *Desulfosarcina* sp.; SB4_53; AY177791	–	0.665
B73	BS	Bv	WIP 10m 11A	uncultured delta proteobacterium; HMMVPog-2; AJ704678	93	0.737
B74	Ya	An	MLSB 6m 3G	uncultured bacterium; KM88; AY216443	–	0.647
				Desulfobacterium indolicum; AJ237607	89	–
B75	Ya	Bk	WIP 20m 8C	uncultured bacterium; KM88; AY216443	–	0.645
				Desulfobacterium cetonicum (T); DSM 7267; AJ237603	95	0.603
B76	I	Bea	WIP 6m 1D	Unidentified bacterium clone Qui4P2-36; AJ518578	97	–
CFB						
B77	I	Boa	WIP 10m 1H	uncultured bacterium; Gitt-KF-184; AJ532698	94	0.765
B78	I	G	#12 - 3C*	uncultured bacterium SHA-107; AJ306739	97	0.786
B79	I	N	#12 - 4C*	uncultured bacterium SHA-107; AJ306739	97	0.789
B80	X	AD	#24 - 9G*	uncultured bacterium PL-38B1; AY570569	99	0.984
Chloroflexi						
B81*	BK	AL	MLSB 20m 12A	uncultured bacterium; 2-3; AY548942	96	0.874
B82	Bpa	Aj	MLSB 6m 7B	uncultured bacterium; FA2; AY553935	98	0.933
Sphingobacterium						
B83	K	Ra	#22 - 4E*	uncultured bacterium, ARKCRY2; AY198110	96	0.865
				Hymenobacter sp. MJ1; AF449431	96	–
Spirochaetes						
B84	AU	Bj	MLSB 20m 1G	uncultured bacterium; E5; AY426470	96	0.903
				Spirochaetes bacterium SA-8; AY695839	92	0.738
Clostridia						
B85	E	E	#12 - 10F*	*Clostridium* sp. 94B; AY554416	95	0.739
B86	E	C	#12 - 1F*	*Clostridium* sp. 94B; AY554416	95	0.744

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity GenBank	RDP
B87	E	A	#12 - 14H*	*Clostridium* sp. 94B; AY554416	95	0.760
B88	C	A	#12 - 1A*	Uncultured bacterium clone PL-35B7; AY570625	98	0.917
				Clostridium sp. 9B4; AY554416	97	0.822
B89	B	A	#12 - 2D*	*Clostridium* sp. 9B4; AY554416	97	0.827
				uncultured bacterium; PL-35B7; AY570625	97	0.910
B90	B	O	#12 - 6A*	Uncultured bacterium clone PL-35B7; AY570625	97	0.906
				Clostridium akagii strain CK58; AJ237755	93	–
B91	B	C	#12 - 8C*	*Clostridium* sp. 9B4; AY554416	96	0.790
				uncultured bacterium; PL-35B7; AY570625	92	0.900
B92	B	K	#12 - 9C*	uncultured bacterium; PL-35B7; AY570625	98	0.909
				Clostridium sp. 9B4; AY554416	97	0.814
B93	B	B	#12 - 1B*	*Clostridium* sp. 94B; AY554416	96	0.796
B94	C	B	#16 - 8G*	Uncultured baceterium PL-35-B7; AY570625	98	0.902
				Clostridium sp 9B4; AY554416	96	–
B95	AD	un1	#22 - 8A*	Uncultured baceterium PL-35-B7; AY570625	97	0.909
				Clostridium sp 9B4; AY554416	97	0.817
B96	S	P	#12 - 17A*	*Clostridium aminobutyricum* DSM 2634; X76161	96	0.863
				Uncultured bacterium Eub No. 20; AF395430	97	0.863
B97	H	H	#12 - 3G*	*Clostridium aminobutyricum* strain DSM 2634; X76161	95	0.788
				Anaerovorax odorimutans strain NorPut; AJ251215	93	0.722
B98	S	H	#12 - 9F*	Uncultured bacterium Eub No. 20; AF395430	96	0.829
				Clostridium aminobutyricum DSM 2634; X76161	95	0.827
B99	H	P	#24 - 3C*	*Clostridium aminobutyricum* DSM 2634; X76161	95	0.818
				Uncultured bacterium Eub No. 20; AF395430	98	0.913
B100	BC	Ar	MLSB 20m 1E	uncultured bacterium; Eub No. 20; AF395430		0.876
				Clostridium aminobutyricum; DSM 2634; X76161	96	0.843
B101	S	Ahb	MLSB 30m 1E	*Clostridium aminobutyricum*; DSM 2634; X76161	95	0.833
B102	AB	H	#22 - 3C*	uncultured bacterium PL-5B1; AY570633	97	0.895
				Fusibacter paucivorans; AF050099	94	0.737
B103	AB	P	#22 - 7A*	uncultured bacterium PL-5B1; AY570633	97	0.902
				Fusibacter paucivorans; AF050099	94	0.749
B104	A	E	#12 - 11B*	*Soehngenia saccharolytica;* AY353956	97	0.894
	A	E	#12 - 2A*	*Clostridium* sp. (BN II); X75909	94	0.741
	A	E	#12 - 6E*	*Soehngenia saccharolytica;* AY353965	95	0.741
B105	A	D	#12 - 1H*	*Soehngenia saccharolytica;* AY353956	94	0.585
B106	A	Z	#16 - 3C*	*Tissierella praeacuta* type strain NCTC 11158; X80832	–	0.765
B107	AD	un2	#22 - 10C*	*Soehngenia saccharolytica* BOR-Y; AY353956	94	–
B108	AA	V	#22 - 4B*	*Acetobacterium halotolerans* SyrA5; AY744449	97	0.929
B109	AA	M	#22 - 4C*	*Acetobacterium halotolerans* SyrA5; AY744449	96	0.913

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OTU#	HaeIII	CfoI	Sequenced Clone	Closest Relative(s)	% Identity	
					GenBank	RDP
B111	AE	Ahb	MLSB 10m 1H	*Desulfotomaculum kuznetsovii*; 17; AY036904	91	0.530
				toluene-deg.methanogenic consort.bact Eub 1; AF423181	94	0.555
B112	AQ	A	MLSB 10m 5D	uncultured bacterium; GIF17; AF407206	94	0.810
				Desulfotomaculum kuznetsovii; 17; AY036904	92	0.594
B113	AE	Aha	MLSB 20m 3F	*Desulfotomaculum thermobenzoicum*; DSM 6193; 11; AJ294430	92	0.507
				uncultured low G+C Gram-positive bacterium; 36-20; AF351221	94	0.551
B114	AO	P	MLSB 6m 6D	uncultured low G+C Gram-positive bacterium; 36-20; AF351221		0.555
				Desulfotomaculum gibsoniae; DSM 7213; 19A; AJ294431	91	0.528
B115	BA	Ahb	WIP 10m 2D	*Sporotomaculum syntrophicum* (T); FB; AB076610	–	0.538
				toluene-degrading meth consort bact; Eub 1; AF423181	94	0.597
B116	J	Q	#12 - 5C*	Uncultured firmicute Clone P. palm C/A 51; AJ441230	94	0.627
				Clostridium tetanomorphum (NCIMB11547); X68184	87	–
B117	A	V	#12 - 11E*	*Carnobacterium alterfunditum;* L08623	99	0.968
B118	G	L	#12 - 4A*	*Carnobacterium alterfunditum;* L08623	99	0.954
Acholeplasma						
B120	A	Ai	MLSB 6m 8H	uncultured bacterium; EUB33-2; AY693836	96	0.829
				Acholeplasma palmae (T); ATCC49389; J233; L33734	90	0.627
B121	ATb	At	MLSB 6m 9B	uncultured bacterium; TANB25; AY667255	96	0.796
				Acholeplasma palmae (T); ATCC49389; J233; L33734	91	0.642
B122	AX	Ah	MLSB 30m 9D	uncultured bacterium; EUB33-2; AY693836	95	0.862
				Acholeplasma parvum; H23M; AY538170	–	0.660
B123	I	AA	#22 - 12B*	Uncultured bacterium KD1-22; AY218558	–	0.775
				Uncultured bacterium clone KD4-26; AY188312	96	–
B124	K	B	#12-7C*	*Acholeplasma axanthum;* AF412968	93	0.633
B125	BR	A	MLSB 6m 3B	*Acholeplasma parvum*; H23M; AY538170	87	0.517
B127	A	Ah	WIP 10m 12F	uncultured bacterium; EUB33-2; AY693836	96	0.817
				Acholeplasma brassicae; 0502; AY538163	92	0.621
E. coli						
B128	Y	AA	#16 - 1D*	E. coli K12 (U18997)	99	0.960
B129	Y	AB	#16 - 1H*	E. coli K12 (U18997)	99	0.932
B130	Y	AA	MLSB 10m 1A	E. coli K12 (U18997)	97	0.985
B131	Y	ALa	WIP 10m 1C	E. coli K12 (U18997)	98	0.993
B132	Y	Ay	WIP 20m 9A	E. coli PK3 (X80728)	99	0.976

* indicates full length sequence was used

Appendix 7:
Physical and chemical properties of MFT sampled from MLSB and WIP. Data provided by Syncrude Canada.

Properties of Waters from Syncrude's Mildred Lake Settling Basin (MLSB).
Collected May 2004 from a Central location*. Surface Elevation 349 m (AMSL)

	Water Zone	Fine Tails Zone							
Depth	**2 m**	**6 m**	**10 m**	**15 m**	**20 m**	**25 m**	**30 m**	**35 m**	**40 m**
General									
Elevation (AMSL) (m)									
pH (units)	7.69	7.37	7.00	7.18	7.54	7.77	7.96	8.06	7.92
Conductivity (uS/cm)	2940	3770	3840	3580	3650	4070	4270	4030	3910
Temperature (ºC)	11.6	12	16.9	19.6	22	23.3	21.2	20.6	20.3
Dissolved Solids (% by wt)**		40.7	42.5	55.6	60.6	57.9	71.6	63.3	73.4
Solids Content (g/100g)	0.248	42.3	43.4	58.2	60.2	61.5	71.8	62.5	74.1
Methylene Blue (mls 6 mN/100g)		1797	1391			586	363	468	302
Alkalinity (mg/L)**									
Chem Oxy Demand (mg/L)**	164	140	139	146	165	185	185	200	180
Biol Oxy Demand (mg/L)	8.9								
Dissolved Oxygen (mg/L)									
Redox Potential (mV)	203	-197	-121	-121	-112	-102	-198	-111	-143
Phenols (mg/L)**	0.03	0.02	0.01		0		0		0
Cyanide (mg/L)**	0.055	0.013	0.007		0.006		0.006		0
Sulfides (mg/L)		0	0		0		0		
Total Pet Hydrocarbons (mg/L)	21								
Bitumen Content (% by wt)		2.57	2.24	2.74	1.68	1.82	1.11	1.37	0.99
Naphtha Content (% by wt)									
Dis. Organic Carbon (mgC/L)**	47	44	44	43	50	52	56	55	55
Naphthenic Acids (mg/L)	52.1	42.3	40.6	42.7	84	83.7	95.3	98.7	82.6
Tannin&Lignin (mg/L)**	3		2		3		3		3
Surfactants (MBAS)** (mg/L)	1		0		0		0		0
Hardness (as CaCO3)									
Toxicity									
IC50 (% by vol)	52	100	100	100	74	63	66	46	25
IC20 (% by vol)	16	40	23	100	22	11	11	9	6
Nutrients									
o-Phosphate (mgP/L)	0.008	0.034	0		0.078		0.098		0.084
Total Phosphorus (mgP/L)									
Ammonia (mgN/L)									
Nitrate+Nitrite (mgN/L)	0.006	0.022	0.029		0.036		0.038		0.026
Total Nitrogen (mgN/L)	20	19	12		9		11		9
Major Ions									
Cations									
Na+	674	888	925	824	923	1040	1140	1110	1040
K+	11.7	21.3	20.9	21	13.9	14.5	15	13.3	13.9
Mg++	10.9	18.4	18	22.6	10.8	7.9	9.5	7.2	7.3
Ca++	18.2	28.8	24.9	30.1	14.7	8.5		7.2	7.5
Anions									
F-	1.8	BDL	BDL	BDL	2.9	BDL	BDL	BDL	BDL
Cl-	460	470	430	530	590	670	713	710	626
SO4=	232	0.1	12.8	14.1	19.6	335	36	36	25.1
CO3=	0	0	0	0	0	0	0	0	0
HCO3=	779	1700	1790	1380	1220	1350	1470	1380	13.7

* Samples collected at Central Station on MLSB in May 2004: 53958N, 51741E (mine metric)

** Fine Tails Pore Water (Centrifuged at 30 000g and 0.45um filter-passing)

SECTION 5.2 (c), 4.2

TRACE ELEMENT CONTENT OF MILRED LAKE SETTLING POND WATERS: 2004**

		Surface Zone ("Free Water")	Fine Tails Zone (Pore Water)						
Depth	**units**	**2 m**	**6 m**	**10 m**	**15 m**	**20 m**	**25 m**	**30 m**	**40 m**
Dissolved* Trace Metals									
Aluminum (Al)	mg/L	0.176	0.068	0.023	0.043	0.042	0.342	0.656	1.07
Antimony (Sb)	ug/L	0.001	0.002	0.001	0.001	0.001	0.001	0.006	0.003
Arsenic (As)	mg/L	0.022	0.003	0.002	0.003	0.049	0.002	0.022	0.004
Barium (Ba)	mg/L	0.318	0.667	0.559	0.477	0.49	0.294	0.28	0.319
Beryllium (Be)	mg/L	0	0	0	0	0	0	0	0
Boron (B)	mg/L	1.83	4.22	4.55	3.16	3.31	3.52	3.32	3.48
Cadmium (Cd)	mg/L	0	0	0	0	0	0	0	0
Calcium (Ca)	mg/L	16.6	25.8	21.3	20.9	13.4	7	7.1	6.3
Chromium (Cr)	mg/L	0	0	0	0	0	0	0	0
Cobalt (Co)	mg/L	0	0	0	0	0	0	0	0
Copper (Cu)	mg/L	0.004	0	0	0	0	0	0	0.001
Iron (Fe)	mg/L	0.16	0.45	0.61	0.74	0.21	0.16	0.22	0.32
Lead (Pb)	mg/L	0	0	0	0	0	0	0	0
Lithium (Li)	mg/L	0.118	0.23	0.282	0.207	0.17	0.18	0.196	0.172
Magnesium (Mg)	mg/L	9.2	14.8	14.2	14.2	8.8	6	6.4	5.6
Manganese (Mn)	mg/L	0.061	0.073	0.066	0.034	0.024	0.017	0.017	0.016
Mercury (Hg)	ug/L	0		0		0		0	
Molybdenum (Mo)	mg/L	0.075	0.003	0.088	0	0.014	0.023	0.098	0.061
Nickel (Ni)	mg/L	0.012	0.01	0.088	0.005	0.006	0.005	0.005	0.006
Potassium (K)	mg/L	10.2	17.4	17.1	13	11.4	11.7	11.9	10.8
Selenium (Se)	ug/L	0.001	0.001	0	0.001	0.001	0	0	0
Silicon (Si)	mg/L	2.37	5.08	6.43	5.22	4.11	4.04	4.27	5
Silver (Ag)	mg/L	0	0	0	0	0	0	0	0
Sodium (Na)	mg/L	540	785	760	680	719	795	922	873
Strontium (Sr)	mg/L	0.58	0.94	0.84	0.76	0.51	0.39	0.42	37
Sulfur (S)	mg/L	74.9	3.1	6.6	3.9	5.8	4.7	10.3	11
Thallium (Tl)	mg/L	0.001	0	0	0	0	0	0	0
Tin (Sn)	mg/L	0.006	0	0	0	0	0	0	0
Titanium (Ti)	mg/L	0	0	0	0	0	0.006	0.006	0.007
Uranium (U)	mg/L	0.006	0	0	0	0	0	0.005	0.003
Vanadium (V)	mg/L	0.016	0	0	0	0	0	0.006	0
Zinc (Zn)	mg/L	0.039	0.042	0.034	0.048	0.035	0.037	0.024	0.027
Zirconium (Zr)	mg/L	0.013	0.01	0.007	0.003	0.004	0.006	0.009	0.01

* Dissolved: 0.45 um filter-passing. Analyzed using ICP/MS (except As, Se, Sb by Hydride AA and Hg by Cold Vapor AA) by Maxxam Analytical. All values expressed in mg/L unless otherwise noted.

** Samples collected at Central Station on MLSB on May, 2004: 5958N, 51741E (mine metric)

MILDRED LAKE SETTLING POND:
Particle Size Distribution of Solids in Fine Tails Zone
Collected from a Central Region of the MLSB on May, 2004

	Fine Tails Zone Depth										
	5 m	6 m	8 m	10 m	13 m	15 m	20 m	25 m	30 m	35 m	40 m
Solids Content (g/100g)	35.1	40.7	41	42.5	50.9	55.6	60.6	57.9	71.6	63.3	73.4
Fines Content (<22um/<22um+w)											
Bitumen Content (g/100g)	3.76	2.57	2.25	2.24	2.8	2.74	1.68	1.82	1.11	1.37	0.99
Methylene Blue** (0.006N mls/100g)		1797		13.91	1132			586	363	468	302
PARTICLE SIZE DISTRIBUTION* (% less than each size fraction)											
720 um	100	100	100	100	100	100	100	100	100	100	100
500 um	100	100	100	100	100	100	100	100	100	100	97
250 um	100	100	100	100	100	100	93	88	82	79	63
125 um	100	100	99	99	99	96	81	72	66	63	44
88 um	99	97	96	96	96	91	75	66	60	57	39
44 um	98	95	92	92	92	86	69	60	55	52	34
22 um	95.4	90	86.9	96.6	85.5	78.6	91.9	53.9	48.8	46.6	30.1
16 um	87.1	78.8	75.8	74.8	72.2	64.9	50.7	43..4	39.8	37.4	23.8
11 um	69.2	61.1	58.6	57.6	53.7	48.2	37.9	32.2	28.3	27.7	17.5
7.8 um	58.5	51.6	49.3	48.5	42.9	39.7	31.3	27	21.9	23.1	14.5
5.5 um	47.4	42.1	39.9	39.3	32.3	31.4	24.8	22	16.1	18.8	11.6
3.9 um	33.9	30.6	28.6	28.1	21.2	22	17.3	16.1	10.5	13.6	8.3
2.8 um	24.2	22.1	20.6	20.1	14.2	15.5	21.1	11.8	7.3	9.9	6
1.9 um	15.4	14.3	13.2	12.8	8.6	9.8	7.6	7.8	4.9	6.5	3.9
1.4 um	8.6	8.1	7.5	7.2	4.7	5.5	4.3	4.6	3.2	3.8	2.3
1.0 um	4.38	4.17	3.93	3.67	2.47	2.85	2.22	2.47	2.132	2.07	1.2
0.7 um	1.77	1.71	1.65	1.5	1.09	1.21	0.95	1.1	1.34	0.94	0.6
0.5 um	0.56	0.55	0.55	0.49	0.41	0.43	0.34	0.41	0.82	0.36	0.2
0.35 um	0.1	0.11	0.12	0.1	0.12	0.1	0.09	0.11	0.47	0.1	0.1
0.25 um	0	0	0.01	0	0.01	0.01	0.01	0.01	0.24	0.01	0
0.18 um	0	0	0	0	0	0	0	0	0.12	0	0
0.1 um	0	0	0	0	0	0	0	0	0	0	0

Samples collected at Central Station on MLSB, May 2004: 53958N, 51741E (mine metric). Surface 349 m (AMSL)

* PSD determined using Coulter Laser Diffraction Method at SCL Research Dept.

** MB by ulrasonic method at MRRT (Fort McMurray)

Properties of Surface and MFT Pore Waters from SYNCRUDE'S West Inpit Pond.
Collected April 20, 2004 from Station 1, WIP*. Surface Elevation 291 m (AMSL)

	Water Zone	Fine Tails Zone						
Depth	**2 m**	**6 m**	**10 m**	**15 m**	**20 m**	**25 m**	**30 m**	**35 m**
General								
Elevation (AMSL) (m)	289	285	281	276	271	266	261	256
pH (units)	8.02	7.75	7.73	7.92	7.9	7.89	7.64	789
Conductivity (uS/cm)	3800	4370	4630	4480	4480	4590	2740	4250
Temperature (ºC)	8.3	11.2	14.2	14.9	15.8	15.6	11.6	12.4
Dissolved Solids (% by wt)**	0.221	0.3	0.31	0.32	0.32	0.33	0.27	0.31
Solids Content (g/100g)	0.118	32.9	36.4	41.7	43.2	44.2	51.8	55.9
Methylene Blue (mls 6 mN/100g)	-	1349	1074	946	874	680	1050	548
Alkalinity (mg/L)**	690	1080	1150	1150	1065	1090	1515	1090
Chem Oxy Demand (mg/L)**	195	235	220	230	230	260	210	260
Biol Oxy Demand (mg/L)	2.5	-	0.9	-	3.1	-	1.3	-
Dissolved Oxygen (mg/L)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Redox Potential (mV)	112	-239	-319	-136	-128	-228	-290	-171
Phenols (mg/L)**	<0.01	<0.01	<0.01	-	<0.01	-	<0.01	-
Cyanide (mg/L)**	<0.005	<0.005	<0.005	<0.005	<0.005	-	<0.006	-
Sulfides (mg/L)	<0.01	<0.01	<0.01	-	<0.01	-	<0.01	-
Total Pet Hydrocarbons (mg/L)	4	-	-	-	-	-	-	-
Bitumen Content (% by wt)		2.45	2.13	2.39	2.36	2.32	2..4	2.4
Naphtha Content (% by wt)		0.02	0.09	0.03	0.04	0.03	0.19	0.03
Dis. Organic Carbon (mgC/L)**	49.9	53.6	53.2	51.9	51	51.3	55.8	55.2
Naphthenic Acids (mg/L)	80.1	69	67.2	70.6	69.2	85.9	72.1	77.8
Tannin&Lignin (mg/L)**	5	-	2	-	2.5	-	2	-
Surfactants (MBAS)** (mg/L)	<0.02	-	0.7	-	0.8	-	0.7	-
Hardness (as CaCO3)	52	60	77	58	58	58	90	78
Toxicity								
IC50 (% by vol)	46	100	100	100	100	100	100	100
IC20 (% by vol)	12	34	21	26	23	19	35	24
Nutrients								
o-Phosphate (mgP/L)	0.048	0.029	0.049	-	0.115	-	0.019	-
Total Phosphorus (mgP/L)	0	0.1	0.3	-	0.5	-	0.1	-
Ammonia (mgN/L)	7	5.8	8.8	4.6	6.1	6.5	10	3.7
Nitrate+Nitrite (mgN/L)	0.031	0.011	0.011	-	0.015	-	0.017	-
Total Nitrogen (mgN/L)	5.78	5.31	7.36	-	5.92	-	9.72	-
Major Ions								
Cations								
Na+	988	1120	1100	1540	1220	1150	975	1040
K+	11.5	12.8	13.7	11.9	12.4	13.8	16.7	14.3
Mg++	6.6	7.8	9.3	7.4	7.1	7.1	11.3	7.4
Ca++	9.5	10.9	14.9	12.2	11.2	11	18	10.9
Anions								
F-	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
Cl-	750	850	850	820	920	900	400	790
SO4=	290	45	70.1	67.8	18.9	36.7	36.5	22.1
CO3=	0	0	0	0	0	0	0	0
HCO3=	849	1320	1400	1390	1300	1330	1850	1380

* Samples from Station 1 in WIP, April 20, 2004: 47077N, 51589 (mine metric). Surface elevation 291m (AMSL)

** Fine Tails Pore Water (Centrifuged at 30 000g and 0.45um filter-passing)

Elemental Content of West In-Pit Waters: April 20, 2004

		Surface Zone ("Free Water")	Fine Tails Zone (Pore Water)						
Depth	**units**	**2 m**	**6 m**	**10 m**	**15 m**	**20 m**	**25 m**	**30 m**	**35 m**
Dissolved* Trace Metals									
Aluminum (Al)	mg/L	0.082	0.11	0.135	0.525	0.47	1.28	4.27	1.57
Antimony (Sb)	ug/L	0.0014	0.0006	0.0009	0.00055	0.0006	0.0008	0.0007	0.0005
Arsenic (As)	mg/L	0.0097	0.0326	0.0189	57	0.0037	0.0074	0.0092	0.0022
Barium (Ba)	mg/L	0.298	0.442	0.514	0.33	0.364	0.305	0.634	0.405
Beryllium (Be)	mg/L	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	0.0003	<0.0002
Boron (B)	mg/L	2.97	4.31	0.397	4.13	3.91	4.18	4.66	4.47
Cadmium (Cd)	mg/L	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002
Calcium (Ca)	mg/L	9.5	10.9	14.9	12.2	11.2	11	18	10.9
Chromium (Cr)	mg/L	0.004	0.004	0.004	0.005	0.005	0.6006	0.012	0.006
Cobalt (Co)	mg/L	0.0037	0.0008	0.0005	0.0006	0.0006	0.0009	0.0017	0.0009
Copper (Cu)	mg/L	0.0024	0.0011	0.0008	0.0009	0.0015	0.0007	0.0017	0.001
Iron (Fe)	mg/L	0.04	0.07	0.08	0.19	0.016	0.41	1.35	0.44
Lead (Pb)	mg/L	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	<0.0003	0.0013	<0.0003
Lithium (Li)	mg/L	0.23	0.278	0.309	0.29	0.286	0.288	0.312	0.278
Magnesium (Mg)	mg/L	6.6	7.8	9.3	7.4	7.1	7.1	11.3	7.4
Manganese (Mn)	mg/L	0.046	0.033	0.029	0.023	0.019	0.022	0.047	0.031
Mercury (Hg)	ug/L	<5E-05	-	<5E-05	-	<5E-05	-	<5E-05	-
Molybdenum (Mo)	mg/L	0.101	0.0196	0.0272	0.0361	0.0392	0.0386	0.0136	0.0316
Nickel (Ni)	mg/L	0.0043	0.0012	<0.0005	<0.0005	<0.0005	<0.0005	0.0027	<0.0005
Potassium (K)	mg/L	11.5	12.8	13.7	11.9	12.4	13.8	16.7	14.3
Selenium (Se)	ug/L	0.0025	0.0007	0.0005	0.0004	0.0004	0.0004	0.0006	0.0006
Silicon (Si)	mg/L	1081	3.94	4.23	4.63	4.15	5.48	14.2	6.72
Silver (Ag)	mg/L	0.0008	0.0006	0.0005	0.0003	0.0004	0.0004	0.0002	0.0002
Sodium (Na)	mg/L	988	1120	1100	1540	1220	1150	975	1040
Strontium (Sr)	mg/L	0.55	0.65	0.79	0.66	0.63	0.62	0.86	0.63
Sulfur (S)	mg/L	88.5	17.3	7.4	20.2	14.5	12.5	14.4	11.3
Thallium (Tl)	mg/L	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002	<0.0002
Tin (Sn)	mg/L	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Titanium (Ti)	mg/L	0.001	0.002	0.002	0.005	0.004	0.008	0.022	0.01
Uranium (U)	mg/L	0.008	0.0037	0.0033	0.0036	0.004	0.0055	0.0012	0.0062
Vanadium (V)	mg/L	0.005	0.002	0.002	0.002	0.004	0.006	0.01	0.004
Zinc (Zn)	mg/L	0.0178	297	0.0342	0.0141	0.0249	0.0167	0.0592	0.026
Zirconium (Zr)	mg/L	0.0077	0.0067	0.0053	0.0059	0.0053	0.0073	0.0154	0.0075

* Dissolved: 0.45 um filter-passing. Analyzed using ICP/MS (except As, Se, Sb by Hydride AA and Hg by Cold Vapor AA) by Maxxam Analytical. All values expressed in mg/L unless otherwise noted.

** Samples collected at Station 1 in West In-Pit on April 20, 2004: 47077N, 51589E (mine metric). Surface elevation 291 m (AMSL)

SECTION 5.2 (c), 4.2

West In-Pit: Particle Size Distribution of Solids in Fine Tails Zone: April 20, 2004

	Fine Tails Zone Depth									
	6 m	8 m	10 m	11 m	13 m	15 m	20 m	25 m	30 m	35 m
Solids Content (g/100g)	30.8	34.4	37	41.4	42.2	42.7	43.2	44.2	51.8	55.9
Fines Content (<22um/<22um+w)	28.8	31.4	32.8	36.6	38.8	34.2	34.6	35.2	43.9	33.7
Bitumen Content (g/100g)	2.45	2.1	2.13	2.29	2.26	2.39	2.36	2.32	2.4	2.4
Methylene Blue** (0.006N mls/100g)	1349	1397	1074	1198	1341	946	847	680	1050	548
PARTICLE SIZE DISTRIBUTION* (% less than each size fraction)										
720 um	100	100	100	100	100	100	100	100	100	100
500 um	100	100	100	100	100	100	100	100	100	100
250 um	100	100	100	100	100	99.9	99.99	99.99	100	99.6
180 um	100	99.99	100	99.8	99.99	98	99.1	99	100	93.6
120 um	100	98.8	99.2	97.4	99	92.5	95.5	95.1	98.6	82.6
88 um	100	96.9	96.9	94.8	97.1	87.9	91.8	91.3	94.9	74.9
62 um	99.8	96	94.8	92.5	95.8	84.2	88.6	88.4	92.4	69.1
44 um	98.3	93.1	91.3	89.3	93.1	79.3	84.3	78.6	88.6	62.4
31 um	94.9	89.9	86.7	84.9	89.5	73.7	78.9	71.7	84.1	55.4
22 um	88	84.5	80.1	78.7	83.6	66.9	72.1	65.4	77.8	48.6
16 um	79.7	78.7	73.6	72.6	77.4	60.7	66	58	71.6	43.3
11 um	68.9	71	65.3	64.8	69	53.7	58.8	50.3	63.3	37.8
7.8 um	58.3	62.1	56.5	56.2	59.5	46.5	51.4	42.5	54.1	32.7
5.5 um	48.3	52.7	47.4	47.2	49.7	39.3	43.5	35.3	44.6	27.5
3.9 um	39.5	43.8	39.1	38.6	40.7	32.7	35.9	28.4	35.8	22.8
2.8 um	31.3	35.4	31.4	30.4	32.4	26.4	28.3	20.3	28	18.3
1.9 um	22.2	25.6	22.6	21.3	23.2	19	19.8	14.5	19.6	13.2
1.4 um	15.7	18.5	16.4	14.9	16.8	13.7	13.8	9.27	14	9.48
1.0 um	9.85	12	10.6	9.29	10.9	8.84	8.56	5.2	9	6.12
0.7 um	5.4	6.83	6.12	5.11	6.13	5.03	4.67	2.67	5.17	3.51
0.5 um	2.69	3.59	3.24	2.59	3.37	2.63	2.34	1.1	2.77	1.86
0.35 um	1.07	1.53	1.4	1.06	1.47	1.12	0.94	0.37	1.23	0.82
0.25 um	0.34	0.53	0.5	0.35	0.54	0.4	0.3	0	0.47	0.3
0.13 um	0	0.01	0.01	0	0.022	0.01	0	0	0.02	0.01
0.1 um	0	0	0	0	0	0	0		0	0

Samples collected at Station 1 in WIP on April 20, 2004: 47077N, 51589E (mine metric). Surface 291 m (AMSL)

* PSD determined using Coulter Laser Diffraction Method at SCL Research Dept.

** MB by ulrasonic method at MRRT (Fort McMurray)